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13. ABSTRACT *(Maximum 200*

Hypothesis: repletion of mild zinc (Zn) and iron (Fe) deficiencies will improve cognition of premenopausal women. Design: double-blind stratified randomized controlled treatment (with cross-over) trial. Subjects (ss): 60 Zn-Fe deficient (D) and 20 normal (N). Treatments (Rx): micronutrients (M) alone given to 20 D and 20 N ss for 16 w, with pseudo-cross-over at 8 w; 30 mg Zn + M & 30 mg Fe + M, each for 8 w, with cross over at 8 w. Outcomes: change in cognition after Rx, relation of cognition to Fe & Zn status, exchangeable Zn pool (EZP), Zn turnover rate (TR), lean body mass (LBM), and some indices of metabolism. Results: mean ± SD serum ferritin = 24 ± 17 ng/mL in 53 ss; plasma (pl) Zn = 618 ± 236.7μg/L in 50 ss; non-extracted (non-X) pl provides more reliable $^{67/68}$Zn data; in 22 ss non-X, pl Zn disappearance constant (K) is 0.0199 ± 0.0021 min^{1}; 24 h EZP is 151.8 ± 33.0 mg; EZP/BMI is 6.6 ± 1.1 mg/kg/m^2; 24 h EZP by isotope dilution & kinetics are similar (r=0.994); EZP correlates with TR & LBM (r = 0.83 & 0.81). Measurements of Rx effects on cognition continue.

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FOREWORD

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Harold K. Jaunstead 12/7/97

PI - Signature Date

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Introduction

Subject: The relationship of zinc and iron nutriture to neuropsychological performance.

Purpose: This project tests the hypothesis: "Repletion of mild zinc and iron deficiencies will improve neuropsychological performance of premenopausal women."

Scope: The project has three major components:

- Premenopausal women who are likely to be mildly deficient in zinc and iron are identified through food frequency histories and measurement of serum ferritin concentrations (1).
- Zinc status is confirmed by plasma, granulocyte, lymphocyte, platelet, urine, and hair zinc concentrations and zinc kinetics. Iron status is confirmed by serum ferritin and iron, percent iron saturation of transferrin, hemoglobin and red blood cell indices.
- Effects of zinc and iron repletion on neuropsychological performance are measured by computerized tasks, using a stratified randomized controlled double-blind cross over design.

Body

Statement of Work

This 3 year project tests the hypothesis: "Repletion of mild zinc and iron deficiencies will improve neuropsychological (neuromotor and cognitive) functions of premenopausal women."

The design is a double-blind stratified randomized controlled treatment trial with a cross-over of treatments. Sixty zinc-iron deficient subjects, and 20 normal control subjects are studied. Twenty of the deficient subjects and the 20 normal subjects are given a micronutrient "placebo" to control for study effects and replete latent deficiencies that might interfere with responses to zinc and iron. For 8 weeks 20 deficient subjects are given 30 mg zinc with micronutrients and 20 are given 30 mg iron with micronutrients, then the treatments are switched. Neuropsychological performance is measured at baseline and after 8 and 16 weeks of treatment. The placebo subjects undergo a pseudo-cross over at 8 weeks. The main outcome is the change in neuropsychological performance. The individual and combined effects of zinc and iron, and unique effects related to the order of treatments are determined. Secondary outcomes include comparison of methods for measurement of rapidly exchangeable zinc pools; comparison of kinetic indices to other indicators of zinc status; measurement of relationships between zinc kinetic indices to other indicators of metabolism; measurement of relationships between zinc kinetics and indicators of iron status; and

measurement of relationships between indices of zinc and iron status to food frequency.

Project Time Line (from the grant application, with comments):

0-90 days	Recruit, interview and hire the physicians assistant and masters level technician (**Note:** These persons were not hired, their work is being done by a physician) Purchase initial equipment and supplies including: Computer for neuropsychological testing Treatments ^{67}Zn tracer for measurements of zinc kinetics Prepare ^{67}Zn tracer for human administration Advertise project Start screening respondents to advertisements
Year 1.	Enroll 30 subjects in treatment trial, measure cognition (**Note:** We had great difficulty recruiting and retaining subjects. The reasons were unclear. Thus the project fell behind schedule)
Year 2	Enroll 30 subjects in treatment trial, measure cognition (**Note:** Recruitment improved substantially. A major factor appears to have been a change in staff. Retention continued to be impaired by the length of the project. Therefore we discontinued the measurement s of zinc kinetics)
Year 3	Enroll 20 subjects in treatment trial, measure cognition Prepare final reports for publication

Subjects Time Line (with Notes):

d 1	Telephone interview respondents to advertisements (**Note:** To date, 416 women have responded to our advertisements)
d 7	Screen respondent (n=150) to qualify for the project (**Note**. To date, 150 respondents have been screened. We use resources of the NIH sponsored Clinical Research Center for screening.) Medical history Food frequency history Physical Examination Screening blood chemistries, urine and fecal examinations Serum ferritin, hemoglobin, RBC indices and sedimentation rate
d 14	Select subject (n=100) based on iron criteria (**Note:** To date, 49 subjects have been selected) Start treatment of latent deficiencies with micronutrients before measurement of zinc kinetics (**Note:** This was done in about 45 subjects. The duration depended on stage of menstrual cycle. We stopped because findings in children suggested micronutrient supplementation might mobilize zinc from relatively sequestered pools such as bone). Now that we are no longer measuring zinc kinetics,

micronutrients are being given for 7-10 days before the baseline measurement of cognitive performance. Our research findings in children indicate that the micronutrient supplement will have little or no effect on cognition.

Schedule admission to the Clinical Research Center

d 21 Admit to Clinical Research Center for zinc and iron status (**Note:** We soon discovered subjects preferred to have this procedure done as an outpatient)

1700 regular supper

1800 begin overnight fast

d 22 0645 begin measurements of zinc kinetics (**Note:** measured in 49 subjects and 6 men)
insert catheters into both antecubital veins

0715 start 24 hour urine collection for total zinc

0718 draw baseline blood for zinc and iron indices

0720 inject 1 mg ^{67}Zn intravenously (**Note:** We used 2 mg ^{67}Zn to increase the signal for the Mass Spectrometer)

0740 take blood for zinc disappearance and turnover rate (**Note.** The collection times are modified to increase data)

Later orient the subject to the neuropsychological tasks

d 23 0715 end 24 hour urine collection and take blood for exchangeable zinc pool
begin 60 minute urine collection for exchangeable zinc pool (**Note.** Additional urine was collected in some subjects, for measurements of zinc kinetics)

0900 discharge with instructions for next phase

d 35 Select subjects (n=80) based on zinc and iron criteria (**Note:** To date, 37 subjects have enrolled in the intervention trial. To speed this process we stopped measuring zinc kinetics)

Randomize the subjects to the treatments (**Note:** To maintain blinding this process is done by an individual who has no role in management of the subjects)

d 40± Measure baseline neuropsychological outcomes on day 8-12 of menstrual cycle (**Note**. Bioelectrical Impedance and taste acuity are measured and blood is collected for add-on studies)

Begin treatment

d 96 After 8 weeks of treatment repeat measurements of outcomes on day 8-12 of menstrual cycle (**Note**. Bioelectrical Impedance and other add-ons are also measured)

Cross-over the zinc and iron treatment groups and pseudo-cross-over the control groups

d 152 After 16 weeks of treatment measure outcomes on day 8-12 of menstrual cycle (**Note**. Bioelectrical Impedance and other add-ons are also measured)

Thank subjects for participation

	Give subjects copy of their medical record
	Give subjects nutrition information re: zinc and iron
	Pay the final compensation to the subjects
Later	Send subjects copies of published reports

Methods:

Subject Recruitment: Advertisements are posted on bulletin boards at UTMB and surrounding Colleges. Ads are also placed in news letters and newspapers. Respondents were initially screened by telephone interview. Potential candidates are invited for detailed screening. After Informed Consent, detailed screening includes collection of medical, demographic and dietary information, physical examination and laboratory assessment to rule out disease and define iron status. Individuals with serum ferritin <20μg/mL or >30μg/mL who have normal hemoglobin concentration and erythrocyte sedimentation rate are eligible for participation if other indices are satisfactory.

Assessment of Zinc Status: Plasma zinc concentrations are measured by Atomic Absorption Spectrometry (AAS) (2) and by Inductively Coupled Plasma-Mass Spectrometry (ICP-MS) (3). Granulocyte, lymphocyte and platelet zinc are measured after cell isolation and digestion by AAS (4-6). Zinc kinetics were measured in 38 subjects by isotope ratio ICP-MS analysis of plasma samples collected from subjects at time intervals after intravenous administration of 2 mg ^{67}Zn (1, 3). All samples were prepared by two methods, extraction of the tracer (7), and non-extraction (below and attachment 2) The methods of sample preparation were compared because of the finding that the liners of the caps of the extraction tubes can retain minute amounts of zinc. We now discard the caps after one use and do not use the glass extraction tubes again. We are repeating all analysis where it appears there might be a problem.

Effect of Zinc and Iron Repletion on Outcomes: This phase of the project is "in progress". Sixty low and 20 normal ferritin subjects are studied in this double-blind stratified randomized 16 week treatment trial. Treatments include a micronutrient "placebo", 30 mg zinc with micronutrients and 30 mg iron with micronutrients. The zinc and iron treatments cross-over after 8 weeks of treatment. Twenty low ferritin subjects are assigned the "placebo", 20 are assigned zinc and 20 are assigned iron. The 20 normal subjects are assigned "placebo". To maintain blinding subject given "placebo" undergo a pseudo-cross-over after 8 weeks of treatment. At baseline the subjects are trained to perform computerized neuropsychological tasks that assess attention, perception, higher cognitive processes, spatial orientation, and psychomotor skills (8, 9). Baseline neuropsychological performance and other outcomes are determined on the 8-12th day of the menstrual cycle, then treatment is started. Follow-up is measured after 8 weeks and 16weeks. After an additional 8 weeks neuropsychological performance will again be tested.

Results and Discussion:

Two-hundred-thirty-six women responded to advertisements and provided screening information by telephone. Eighty-nine phone respondents met the criteria for detailed screening. Forty-six respondents met the criteria to be inclusion in the study; their zinc kinetics were measured. After zinc kinetics 31 subjects were enrolled in the treatment trial. Twenty-two completed the first 8 weeks of treatment, and 15 completed 16 weeks of treatment and the follow-up tests.

Review of progress suggested the length of the study impaired recruitment and retention of subjects. Dropouts occurred because subjects moved to another city, lost interest, had irregular menstrual periods or took medications or supplements. Measurements of zinc kinetics were stopped (with permission of the funding agency). This will facilitate achievement of the primary research goal in a timely manner.

The project was audited for use of human subjects by Mrs. C. Smith, from the US Army Medical Research and Material Command and Mrs. J. LeSage, from the Clinical Investigator Regulatory Office on March 6, 1997. Our procedures were judged satisfactory.

Screening of candidates revealed some instances in which serum iron and the percent saturation of transferrin were low but serum ferritin and erythrocyte sedimentation rate were normal. Such individuals were not included. Other individuals were found with adequate indices of iron status, while their dietary history suggested a low intakes of bioavailable dietary iron and zinc. Such individuals were not included.

For 53 subjects the mean ± SD, median and range of serum ferritin were 24 ± 17, 18, and 6-78 ng/mL. Thus subject identification was be successful. These 53 subjects represent 12.7% of the total respondents to advertisements, and 35% of the total respondents who underwent detailed screening. In the US population 25 % of premenopausal women display serum ferritin concentrations <14 ng/mL (10)

Zinc concentrations were measured by AAS in plasma from 50 subjects that was collected between 0700-0800 hours after an over-night fast (11). Mean ± SD, median, and range were 618 ± 236.7, 716, and 158-939 μg/L. The accepted lower limit of normal for fasting adult plasma is 700 μg/L (12). Thus the median value was near the lower limit of normal. We will confirm these data by using results of the ICP-MS analysis. These findings are similar to our earlier observations in premenopausal women (1, 13) and are consistent with the fact that many premenopausal women select diets that are low in bioavailable zinc (14).

Leukocyte and platelet zinc concentrations were measured in 25 mL of whole blood collected from 0700-0800 hours after an over night fast (6) (Table 1). Considerable variation was encountered in the yield of lymphocytes and granulocytes in spite of the fact that the isolation procedure is highly standardized and is reproducible at its various steps. Investigators at other institutions have found similar variations. The reference

ranges cited are based on review of several reports. The wide range suggests there is no consensus. These data will be sorted by ferritin, plasma zinc, and zinc kinetics status when the intervention trial is completed.

Table 1. Leukocyte and Platelet Zinc Concentrations

	Platelets ($ug/10^{10}$ cells)	Lymphocytes ($ug/10^{10}$ cells)	Granulocytes ($ug/10^{10}$ cells)
n	49	45	34
Mean	4.4	96.2	64.3
SD	1.6	37.9	33.5
Median	4.2	91	50.7
Range	2.2-9.4	35.3-254.5	27.5-167.4
Reference values	3.0 - 6.6	45.0 - 218.0	37.8 - 117

The 24 hour urinary zinc excretion was measured in 31 subjects. The mean ± SD and median were 565 ± 317 and 590 μg/L.

Hair zinc concentrations were measured in 49 subjects. The mean ± SD, median, and range were 176.5 ± 79.7, 154.5, and 66-407 μg/g.

Zinc kinetics were measured in 49 subjects and 6 men since the start of this project. Because contamination of extracted samples occurred, we are now analyzing all samples using the non-extraction procedure described below, and repeating all questionable analysis by the extraction procedure using new glassware. Samples from 42 subjects have been analyzed without extraction. Calculations are complete on 22. For them the mean ± SD, median and range of the plasma zinc disappearance constant are 0.0199 ± 0.0021, 0.02, and 0.0163-0.0251 min^1. Their mean ± SD, median, and range of the 24-hour exchangeable zinc pool (EZP) is 151.772727 ± 33.0049517, 149, and 102-240 mg, and the mean ± SD, median, and range of the EZP/Body Mass Index is 6.596 ± 1.105, 6.491, and 4.319-8.239 $mg/kg/m^2$. The relation of these findings to indices of iron status and other indices of zinc status will be determined when all analysis are completed. In our previous study the plasma zinc disappearance constant was highly and inversely related to the serum ferritin concentration (1).

Our zinc kinetic findings are the basis for three reports: (1) Comparison of Tri-Exponential and Truncated Models of Zinc Kinetics; (2) Description of Polyatomic Interferences affecting Direct Analysis of Plasma ^{67}Zn (without extraction) by ICP-MS; and (3) Relationships between Zinc Kinetics and Body Composition.

(1) Comparison of Tri-Exponential and Truncated Models of Zinc Kinetics:
The dilution of ^{67}Zn tracer 24 hours after intravenous administration was shown to be a valid measure of the "rapidly exchangeable zinc (Zn) pool" (EZP) by demonstration of concordance of 24-hour Zn kinetics with 9-day Zn kinetics. Zn kinetics were calculated by 6 methods. Zero-nine-day data from 5 men and 1 woman were used for the calculations. Background Zn IR was subtracted from the plasma Zn IR, then divided by the natural Zn IR to give the normalized IR (NIR). The tri-exponential function K_1 exp(-

g_1 t) + K_2 exp(-g_2 t) + K_3 exp (-g_3 t), where t is time in days, explained (R^2= 0.99) the NIR from 0-9 days. Since the change of the third term during 24 hours was about 10 %, the NIR from 0-24 hours was fitted (R^2 = 0.99) by the above function when g_3 was constrained as 0. The estimated coefficients from the 9-day and 24-hour kinetic models were similar except for g_2. The sum of three pools as a norm of EZP was calculated from the 9-day data using mammillary and catenary 3-pool models with a single outlet, which account for the loss of tracer from the system and the quasi-equilibrium between pools. Thus the mathematical equivalency of the sum of three pools between the mammillary and catenary models was shown, indicating that the EZP is invariant. The 24-hour plasma Zn pool determined by analysis of a single sample of plasma and calculation of isotope dilution was highly correlated (r^2=0.974) with the sum of three pools. Turnover rate was shown to be accurately estimated from the 5-minute and 15-minute data after injection of the isotope tracer (attachment 1).

(2) Description of Polyatomic Interferences affecting Direct Analysis of Plasma ^{67}Zn (without extraction) by ICP-MS: Polyatomic interferences of matrix ions at zinc isotopic masses 64, 66, 67, 68, and 70 were determined by subjecting "simulated human plasma" solutions of single or mixtures of common mineral elements in plasma (S, Na, Cl, K, P, and Ca) to IR ICPMS analysis. The mixture of all 6 mineral elements interfered with ^{64}Zn and ^{70}Zn. Interferences with ^{66}Zn, ^{67}Zn, and ^{68}Zn were minimal. The S-Cl and Na-Cl mixtures decreased the interference with ^{67}Zn. The findings suggested that Na or S affected the chemical reaction of Cl in argon plasma. For the comparison extracted and non-extracted plasma samples, 5-minute to 24-hour plasma samples from 10 subjects, and 5-minute to 9-day samples from 4 subjects were studied. Plasma was digested in hydrogen peroxide and dissolved in nitric acid. Zn in the digestate was extracted into CCl_4 using diethylammonium diethyldithiocarbamate, followed by the extraction of Zn into nitric acid. The solution was heated overnight at 80°C to remove the CCl_4, and dissolved in high purity water to 10 mL after adding yttrium (Y) internal standard. To measure effects of non-extraction, the Y standard and high purity water were added directly to the digestate. After subtraction of the background IR the plasma Zn IR was divided by the natural Zn IR to give the normalized IR (NIR). The logarithmically transformed NIR was calculated for regression analysis. NIRs $^{67/66}$Zn and $^{67/68}$Zn obtained from the extracted samples agreed well (r^2 = 0.998). The NIRs obtained from $^{67/66}$Zn and $^{67/68}$Zn by both the methods agreed well compared to those from other ratios ($^{67/64}$Zn, r^2=0.838; $^{67/66}$Zn, r^2 = 0.976; $^{67/68}$Zn, r^2=0.985; $^{67/70}$Zn, r^2=0.747). Considering the minimum possibility of isobaric interferences in plasma samples, $^{67/68}$Zn obtained from nonextracted samples is satisfactory for measurement of Zinc Kinetics (attachment 2).

(3) Relationships between Zinc Kinetics and Body Composition: Relationships between Zinc Kinetics and body composition have not been reported. The "rapidly exchangeable" zinc pool (EZP) and turnover rate (TR) were measured. Lean body mass (LBM) was determined from the bioelectrical impedance of the subjects. Skeletal muscle mass (SMM) was estimated from the 24-hour creatinine excretion. EZP from the 24-hour kinetic model (above) and by isotope dilution calculation using the 24-hour-spot urine zinc isotope ratio were identical (r=0.994, p< 0.0001). EZP was correlated

the 24-hour kinetic model (above) and by isotope dilution calculation using the 24-hour-spot urine zinc isotope ratio were identical ($r=0.994$, $p< 0.0001$). EZP was correlated with the TR, SMM, LBM and fat mass ($r= 0.83$, $p< 0.0001$; $r= 0.900$, $p< 0.0001$; $r= 0.81$, $p< 0.0001$; and $r= 0.646$, $p= 0.01$). TR was correlated with LBM and SMM ($r= 0.87$, $p< 0.0001$; and $r= 0.82$, p, 0.0001). These finding suggest skeletal muscle is an important source of exchangeable zinc (attachment 3).

In addition to the above plasma beta-hydroxybutyrate concentrations were measured in 104 specimens. All were within the normal range. We assessed this index because research zinc deprived rats found increased plasma beta-hydroxybutyrate concentrations (15) and findings in zinc deprived humans suggested a decrease in the Respiratory Quotient (16).

Folic acid, vitamin B_{12}, pyridoxine, and homocystine are being measured in blood before and after treatment to determine effects of repletion of zinc status on these nutritional indicators. These assays are being done at no cost to this project by T. Tamura, MD of the Department of Nutritional Sciences at The University of Alabama.

Plasma amino acids are being measured to ascertain if zinc treatment affects these indices. Zinc is essential for amino acid utilization. Therefore it is of interest to determine if mild zinc deficiency alters the serum amino acid pattern. The analysis are being done by Richard Fritz, PhD of the Department of Human Biological Chemistry and Genetics at UTMB. These data will be examined when the intervention trial is finished.

Indices of calcium metabolism are being measured by David Simmons, PhD of the Department of Surgery at UTMB to determine effects of zinc repletion on 24-hour urine and plasma calcium, phosphate, osteocalcin, and parathormone. Zinc is essential for bone calcification. Therefore it is of interest to determine if low zinc nutriture affects bone metabolism of young women, who in later life may be at risk of osteoporosis. These data will be examined when the intervention trial is finished.

The effect of zinc repletion on blood cytochrome P450 activity after oral administration of chlorzoxazone is being measured by Douglas Goeger, PhD of the Department of Preventive Medicine and Community Health at UTMB. A P-450 enzyme in esophageal mucosa is implicated in pathogenesis of cancer. When zinc nutriture is low, activity of the enzyme is increased and certain carcinogens are activated(17). The data from this study will be examined when the intervention trial is finished.

Measurements of effects of the treatments on opioid peptides and other hormones by Sam Bathena, PhD of the USDA were planned, but have been discontinued because of an administrative restructuring of his laboratory. Zinc plays a critical role in neurotransmission. It is unfortunate that Dr. Bathena's mission was changed.

Conclusions:

Plasma zinc concentrations of half of the subjects were below the lower limit of normal. This is consistent with our theory and our previous findings.

The method of preparation of samples for ICP-MS analysis $^{67/68}$Zn ratio was improved to minimize the possibility of trace contaminations of samples with isotopic tracers.

The 24 hour exchangeable zinc pool (EZP) and zinc turnover rate were highly related to lean body mass. These findings are original with us and represent an advance in basic knowledge.

The double blind randomized controlled repletion trial is progressing.

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Local:

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Sadagopa Ramanujam VM, Yokoi K, Egger NG, Dayal, HH, Alcock, NW, Sandstead, HH Simplified pretreatment method for plasma samples applicable to zinc kinetic studies. Poster #8, 9th Annual Sigma Xi Research Forum, The University of Texas Medical Branch, Galveston, TX, February 20, 1997.
Egger NG, Yokoi K., Sadagopa Ramanujam VM, Dayal, HH, Alcock, NW, Sandstead, HH Relationship between body composition and rapidly exchangeable zinc pool in humans. Poster #10, 9th Annual Sigma Xi Research Forum, The University of Texas Medical Branch, Galveston, TX, February 20, 1997.

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International:

Yokoi K., Sadagopa Ramanujam VM, Egger NG, Dayal HH, Alcock NW, Sandstead HH Comparison of tri-exponential and truncated models in plasma zinc. *Journal of Investigative Medicine*, 44 (7): 355A, 1996. Poster presented at the 1997 annual meeting of the Japanese Nutrition Society.

Egger NG, Yokoi K., Sadagopa Ramanujam VM, Dayal, HH, Alcock, NW, Sandstead, HH Relationship between body composition and rapidly exchangeable zinc pool in humans. Poster presented at the American Society of Clinical Nutrition annual meeting, July 24-26,1997, Montreal, Canada (attachment 4).

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Comparison of Tri-Exponential and Truncated Models in Plasma Zinc (Zn) Kinetics.

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Abstract

Twenty-four-hour zinc (Zn) kinetics concordant with the nine-day kinetics was developed to show the validity of the 24-h spot plasma Zn pool as a practical indicator of so-called rapidly exchangeable Zn pool size (EZP). We compared kinetic parameters calculated by 6 methods after iv dose of ^{67}Zn derived from 0 - 9 days in six subjects (5 men and 1 woman, age 24 - 64 y, BMI 23.2 - 30.4). Plasma Zn isotope ratios (IR) were measured by inductively coupled plasma - mass spectrometry. After baseline subtraction the plasma Zn IR was divided by the natural Zn IR to give the normalized IR (NIR). The tri-exponential function explained ($R^2 = 0.99$) NIR from 0 - 9 days = $K_1 \exp(-g_1 t) + K_2 \exp(-g_2 t) + K_3 \exp (-g_3 t)$, where t is time in days. Since the change of the third term during 24 hours was about 10 %, NIR from 0 - 24 hours was fitted ($R^2 = 0.99$) by the above function when g_3 was constrained as 0. The estimated coefficients from the 9-d and 24-h kinetic models were similar except for g_2. The sum of three pools as a norm of EZP was calculated from the 0 - 9 day data using the three-pool models (mammillary and catenary); with a single outlet, which account for the loss of tracer from the system and the quasi-equilibrium between pools. We have shown the mathematical equivalency of the sum of three pools between the mammillary and catenary models, indicating that EZP is invariant by other models. The one-day spot plasma Zn pool was highly correlated ($r^2=0.974$) with the sum of three pools, suggesting that the one-day spot plasma Zn pool is a practical indicator of EZP. Turnover rate can be estimated from the initial two points (5 and 15 minutes) after iv dose of ^{67}Zn.

Introduction

Zinc (Zn) is essential for many biochemical functions including protein synthesis and nucleic acid metabolism. It serves as a catalytic component of over 300 enzymes and as a structural component of various proteins, hormones, and nucleotides [1]. Human Zn deficiency occurs worldwide [2-4]. Biochemical indices for evaluating Zn status are imperfect and the specificity of physiological indices is unknown. Zn kinetic parameters have been measured by others using radioactive Zn. Prasad et al [5] used ^{65}Zn to show rapid disappearance of plasma Zn in growth stunted adolescents. Using ^{69m}Zn and ^{65}Zn Aamodt et al [6, 7] observed the change of Zn kinetics after oral loading of 100 mg Zn. Foster et al [8] and Wastney et al [9] developed an integrated Zn kinetic model using ^{65}Zn and ^{69m}Zn.

Stable Zn isotopes are alternatives. Wastney et al [10] compared the results obtained from ^{65}Zn and ^{70}Zn using neutron activation analysis. Miller et al [11] used stable Zn isotopes and fast atom bombardment mass spectrometry [12] for measuring Zn pools. Using a quadrapole inductively coupled plasma - mass spectrometry (ICP-MS), Lowe et al [13] analyzed the 120 min kinetics with ^{70}Zn and Yokoi et al [14-16] evaluated Zn disappearance from 30 to 60 min after an iv dose of ^{67}Zn. Fairweather-Tait et al [17] measured Zn pools using ^{70}Zn and thermal ionization mass spectrometry (TIMS). Scott and Turnlund [18] adapted Wastney's approach [9] to ^{67}Zn and ^{70}Zn tracer using TIMS.

There are two mathematical approaches. 1. The deconvolution method [8, 9] which treats remaining Zn tracer in plasma as a forcing function in the convolution integral [19]. 2. The conventional compartment method based on coefficients in the polyexponential function fitted to the remaining tracer in plasma [20]. The kinetic parameters including the number of exponential terms depend on the observation intervals. We therefore investigated a short-term kinetic model concordant with the long-term kinetic model using ^{67}Zn and Quadrapole ICP-MS.

The rapidly exchangeable zinc pool (EZP) is believed to represent the metabolically active form of zinc which relates to its physiological function [9, 11]. However the definition of EZP is not clear. We report the mathematical equivalency of the sum of three pools between the mammillary and catenary models [21], indicating that the sum of three pools is a well-defined invariant estimate of EZP.

Subjects and methods

Human subjects

Five apparently healthy men and 1 healthy woman living in Texas were the subjects for the 9-day observation (Table 1). This study was approved by the Institutional Review Board at the University of Texas Medical Branch and written consent was obtained from each the subject.

Vials containing 2 mg ^{67}Zn in saline were prepared as reported before [16, 22]. The solutions in the vials were tested for sterility (UTMB Department of Clinical Microbiology and Immunology) and pyrogenicity (Scientific Associates Inc., St. Louis, Missouri).

After a 10 hour overnight fast short Teflon catheters were placed in both antecubital veins of the subjects. The catheters were attached to a slow drip of normal saline by a three-way-stop-cock. A baseline blood sample was taken for the $^{67/68}$Zn ratio. Then 2 mg of sterile, pyrogen free, ^{67}Zn dissolved in normal saline was administered over three minutes through the stop-cock. This was followed by rapid drip of saline for 1 minute. Blood samples were taken from the other catheter at 5, 15, 30, 40, 50, 60 and 90 minutes, 2, 6, 12 and 24 hours, and (2), 3, 5, 7 and 9 days after the administration of ^{67}Zn. Amounts of blood taken at each time point were at least 10 mL. Before each blood collection, about 2 ml blood was taken in a plastic syringe to wash out remaining saline from the catheter. Blood samples were taken in a Monovette syringe containing lithium heparin (10 U/mL blood) obtained from Sarstedt. A 24 hour urine specimen was collected for $^{67/68}$Zn ratio. After emptying the bladder in the morning, 1 hour spot urine samples were collected 2, 3, 4, 5, 6, 7, 8 and 9 days after the iv dose of ^{67}Zn. Blood samples were placed in an ice chest during the collection and promptly delivered to the laboratory for processing.

Laboratory wares and Reagents

The enriched ^{67}Zn (as oxide, purity 93%) was purchased from Oak Ridge National Laboratory, Martin Marietta Energy Systems, Inc., Oak Ridge, TN. Hydrogen peroxide (30%, ultrapure), nitric acid (ultrapure double distilled from Vycor), ammonium hydroxide and hydrochloric acid (ACS grade) were purchased from GFS Chemicals, Ohio. Absolute ethanol was obtained from Fisher Scientific Co, Pittsburgh, PA. Carbon tetrachloride (ACS grade), 2, 6 - dinitrophenol, and Zn and yttrium reference standard solutions were purchased from Aldrich (St. Louis, Missouri, USA). Diethylammonium diethyldithiocarbamate was obtained from Tokyo Kasei, Co., Tokyo, Japan. Deionized water for dilution of the samples was prepared using a Milli-Q

system (Millipore Corp, Milford, MA, USA). Argon gas (99.9% high purity grade) was provided to the ICP-MS from a liquefied argon cylinder (Tri-Gas Industrial Gases, Freeport, TX, USA) capable of delivering at least 20 liter/min at a pressure of 80 psi. The carbon tetrachloride extraction of Zn from the digestate was carried out in borosilicate glass tubes (Kimax, Owens-Illinois Inc., Toledo, Ohio, USA). Disposable Falcon polypropylene tubes (15 mL capacity) used for making multiple dilutions of the digestates were purchased from Fisher Scientific Co, Pittsburgh, Philadelphia, USA. Polypropylene sterile tubes (29 x 114 mm size with red caps) free from Zn contamination used for plasma digestion, were purchased from Sarstedt Inc., Newton, North Carolina, USA.

Chemical analysis

The analysis of isotope ratio (IR) $^{67}Zn/^{68}Zn$ in samples was performed according to previous studies [16, 22]. Zn in the digestate was extracted into the carbon tetrachloride layer as a diethylammonium diethyldithiocarbamate chelate and back-extracted into the diluted nitric acid layer. The purified samples were analyzed by ICP-MS.

Calculation of the normalized isotope ratio

After baseline subtraction, the plasma Zn IR was divided by the natural Zn IR to give the normalized IR (NIR). The spot pool size was calculated as follows:

Spot pool size = Dose of *iv* tracer / NIR / Natural abundance of ^{67}Zn

Mathematical analysis

Mathematical analysis of the data involved three phases. The initial phase was the development of a mathematical model to explain the disappearance of ^{67}Zn from plasma following a single intravenous administration during the restricted observation period. In short, this phase determined the number of exponential terms for the shorter observation period (24 h) concordant with the longer observation period (28 d). In addition, this phase estimated the stability of the nonlinear regression using the Monte Carlo simulation.

The second phase of the mathematical analysis involved the determination of invariant kinetic parameters against various connections of pools, i.e., mammillary and catenary models.

The third phase of the mathematical analysis involved the application of the model to the analysis of data obtained from human subjects.

All modeling was done on a Macintosh Powerbook 165C (Apple) using the SYSTAT 5 for Macintosh, version 5.2.1 software (SYSTAT, Inc., Evanston, IL). A logarithmic transformation of the normalized isotope ratio was used to stabilize the random variation at fitting a polyexponential function to the disappearance data [23].

RESULTS

The first phase of the mathematical analysis

Formulation

The disappearance of Zn tracer from plasma is considered to be explained by a four-exponential function [5, 11] as follows:

$$\textit{Tracer in plasma} = K_1 e^{-g_1 t} + K_2 e^{-g_2 t} + K_3 e^{-g_3 t} + K_4 e^{-g_4 t}$$

$$\textit{where} \quad g_1 > g_2 > g_3 > g_4$$

If $g_1 >> g_2 >> g_3 >> g_4$ as generally found in zinc kinetics [11], the truncated form of the polyexponential function (the bi- or tri-exponential function with a constant term) can be substituted for the complete form when the observation period is shorter than the half-life of the third term. For curve fitting, the truncated form should be used in stead of the complete form to avoid hyper-parameterization. In extreme cases, hyper-parameterization often causes inconvergence found in the nonlinear regression.

According to Miller et al's [11] model based on Wastney et al's report [9], g_1, g_2, g_3 and g_4 are 137.6, 3.564, 0.1106 and 0.00232, respectively. The corresponding half-lives are 7.25 min, 4.67 h, 6.26 d and 298.7 d. We therefore propose the truncated polyexponential model as follows:

For within 24 h, $\textit{Tracer in plasma} = K_1 e^{-g_1 t} + K_2 e^{-g_2 t} + K_3$

For within 28 d, $\textit{Tracer in plasma} = K_1 e^{-g_1 t} + K_2 e^{-g_2 t} + K_3 e^{-g_3 t} + K_4$

Table 2 shows the comparison of the values in Miller et al's model and our estimated parameters from the 5 min - 24 h values using the bi-exponential function with a constant term. The estimates agreed well with the model values, although the consideration is required that the above estimation is based on the calculated values from the model without analytical noise.

Table 3 shows the result of the Monte Carlo simulation. The simulation suggests that 1-2% CV in the measurement of the remaining tracer is acceptable to estimate the kinetic parameters if the Zn tracer disappears according to the four-exponential function described by Miller et al [11].

These investigators [11] developed a four-pool based on Wastney et al's study [9]. We also analyzed in plasma of normal subjects the mean remaining tracer after intravenous administration of ^{65}Zn reported by Wastney et al [9]. For the data obtained from 0 to 28 days and 0 to 2 days, the truncated polyexponential functions are applied to fit the curve. Because Wastney et al did not report the data one day after administration of tracer, the two-day data were utilized. The value from 0 to 290 days was analyzed with the complete quadri-exponential function as a standard. The proportion parameters K_4 (for the term with 158 d of half-life) and K_3 (6.66 d) were respectively predicted from the 28 day and 2 day observations, which were shorter than their corresponding half-lives (Table 4).

The second phase of the mathematical analysis

Calculation of the kinetic parameters in the mammillary model

Based on Landaw et al [24], the kinetic parameters in the mammillary model were calculated. Accepting the single outlet assumption as proposed by Miller et al [11], all parameters in the mammillary model are uniquely determined (See Figure 1; **Appendix 1**). If there are several outlets, only fluxes between pools are uniquely determined [24].

Definition of EZP

Jackson et al [25] and Miller et al [11] defined rapidly exchanging pools of zinc or rapidly exchangeable Zn pools (EZP) as a composite of pools of Zn that exchange completely with plasma within 2 days. Because the system is open to the outside, the system does not reach true isotopic equilibrium but can be in isotopic quasi-equilibrium for certain time intervals (**Appendix 2**). The extent of isotopic equilibrium (or tracer/tracee equilibrium in a broad sense), i. e., the extent of the mixing is evaluated using time vs the ratio of the isotopic enrichments between two pools. The ratio of isotopic enrichments in pool b to pool a (IER_b/IER_a) reaches a maximum value of 1.426 at t = 0.066 day and a 95 % maximum at t = 0.200 day. The ratio of isotopic enrichments in pool c to a (IER_c/IER_a) reaches a maximum value of 1.135 at t = 2.752 day and a 95 % maximum at t = 1.130 day. The ratio of isotopic enrichments in the composite of pools a, b and c to pool a (IER_{a+b+c}/IER_a) takes a maximum value of 1.113 at t = 2.718 day and a 95 % maximum at t = 1.040 day. It is reasonable to

conclude that pools b and c are 'completely' exchanged with pool a (plasma compartment) within 2 days. It is favored that at t = 1 day the ratio of isotopic enrichments in the composite of pool a, b and c to pool a (IER_{a+b+c}/IER_a) is 1.049 which is close to 1 (true complete exchange). The remaining tracer in the composite of pools a, b and c at t = 1 day is 81.2 %. This result supports the hypothesis that the 24 hour spot plasma pool gives a good estimation of EZP.

Possible methods estimating EZP

Obtaining the "true" EZP requires continuous monitoring of isotope ratios in plasma after tracer administration until infinity using the infinite number of terms in the polyexponential function. This method is ideal but impossible to realize. However, an estimation obtained from a long observation period and a polyexponential function with multiple terms approaches the true EZP [26, 27]. Frequent initial sampling and longer observation periods impose a severe limitation on the application of the tracer technique to human zinc metabolism. The first point was discussed by Miller et al, who stated the limitation of the application to children. The second point limits the experimental design and does not allow any change of dietary regimen or another condition during the observation period for the kinetic analysis, which presupposes the steady state. There is a need for a method for estimation of EZP from a shorter observation period and less sampling. The following is a proposed method to estimate EZP.

Method 1 (open three-pool): A sum of three pools in the open three-pool system (mammillary or catenary) calculated from the 9-day observation interval using a tri-exponential function model, which is considered as norm because it has the longest observation period and requires most frequent sampling.

Method 2 (closed three-pool): A pool calculated from the reciprocal of K_3 in the truncated exponential function model applied to the 24-hour observation period, which is equivalent to the sum of three pools in the closed three-pool system (mammillary or catenary).

Method 3 (constrained open-three pool): A sum of three pools in the open three-pool system (mammillary or catenary) with the parameter restriction that fixes g_3 at 0.120 (the empirical value) calculated from the 24-hour observation interval using a tri-exponential function model.

Method 4 (last term of tri-exponential function): A pool calculated from the reciprocal of K_3 of the third term in the tri-exponential function model.

Method 5 (simple extrapolation of Miller et al [11]): A pool calculated from the reciprocal of the intercept obtained from 3 to 9 day extrapolation to the time of the tracer administration (t = 0) using a simple exponential function.

Method 6 (24-hour spot plasma pool): A pool calculated from the reciprocal of the normalized isotope ratio in the spot plasma 24 hours after intravenous administration of tracer.

Considering the pool estimated from the open three-pool method (method 1) as norm, other methods approximate EZP. The restricted open-three pool method (method 3) is essentially a correction of the closed-three pool method (method 2) using the empirical average value of g_3. The restricted open three-pool method is tentative because g_3 might vary more than expected when the subject is in an abnormal condition. The first and the second terms are negligible from 3 days after the administration of the tracer, because g_1 and g_2 are larger than g_3. The last term method (method 4) utilizes K_3 of the third term calculated from the 5 minutes to 9 days data. The simple extrapolation method (method 5) of Miller et al [11] approximates K_3 of the third term method from the 3 to 9 day data. The one-day spot plasma pool method (method 6) is the simplest method that requires just two samples, i.e., one day spot and baseline plasma and approximates the closed three-pool method.

Conserved parameters by matrix transformation of three pool systems of mammillary and catenary models

Because there is no theoretical or experimental basis that justifies the mammillary model as a "true" model, the catenary model is also possible for a three-pool system (Figure 1). Therefore, we investigated the invariant kinetic parameters over different models based on Ramakrishnan's matrix transformation [21]. His basic idea was derived from Berman's model [21]. As was proven in the **Appendix 3**, the following kinetic parameters are invariant:

1. Pool size of the central compartment (plasma Zn pool)
2. Sum of the rate constant from the central compartment (initial slope)
3. Flux from the central compartment (plasma Zn turnover rate)
4. Sum of the pool sizes (so-called rapidly exchangeable Zn pool, EZP).

In the following section, we will limit the discussion within the invariant parameters.

The third phase of mathematical analysis: Application to the analysis of data from the human subjects

Figure 2 shows the illustration of curve fitting of the nine-day data using tri-exponential function model. Table 5 shows the determined coefficients for the tri-exponential model for 9 days data and the truncated model (bi-exponential function with a constant) for 1 day data and the percent deviations of the coefficients of the truncated model from the tri-exponential model. The coefficient g_3 determined from the 9 days data using the tri-exponential model are

less changeable compared to other coefficients (CV: 19 % for K_1; 18 % for g_1; 27 % for K_2; 13 % for g_2; 19 % for K_3; 11 % for g_3). Except for g_2, the determined coefficients from the different model (i.e., different observation period) were similar (Mean of the percent deviation: 2.7 % for K_1; 3.9 % for g_1; 8.9 % for K_2; 37.7 % for g_2; 4.8 % for K_3).

Table 6 shows the indicators describing quasi-equilibrium in the open mammillary system. The average values of the indicators that describe the quasi-equilibrium between central compartment and rapidly exchanging pools are similar to the indicators found in Miller's mammillary model, except for the time when IER_{1+2+3}/IER_1 reaches maximum. The notation of the subscripts '1, 2 and 3' of 'IER' for the subjects correspond to 'a, b and c' for Miller's mammillary model that uses four compartments rather than three compartments.

Table 7 shows the comparison of rapidly exchanging Zn pool (EZP) determined by various methods and the percent deviation of various calculated EZPs estimates relative to Method 1. Considering EZP determined from Method 1 (open three-pool model), overestimation was obvious in the estimates of EZP from Method 2 (closed three-pool model), Method 4 (last term of tri-exponential function), Method 5 (3 - 9 day extrapolation) and Method 6 (one-day spot plasma pool). Method 2 that constrained g_3 as 0.120 in the open three-pool model corrected the overestimation found in Method 2, that constrained g_3 as 0 in the open three-pool model because the closed three-pool model is mathematically a special case of the open three-pool model.

The mean percent deviation was 18 % for Method 2, -11 % for Method 3, 23 % for Method, 44 % for Method 5, 19 % for Method 6. Although Method 3 was aimed to correct the overestimation, the correction was too larger. For Method 5 (3 to 9 day extrapolation) overestimation was larger than other methods. The percent deviation for subject 1 by Method 5 was much larger (66 %) than was observed in another subject. This might be due to the selected interval for simple regression analysis. When the data 2 days later was included (2 - 9 day extrapolation), estimated EZP was 242 mg and the slope was 0.107 day^{-1}. The slope was 0.086 day^{-1} for 3 - 9 day extrapolation, which was smaller than 0.1198 day^{-1} for g_3.

The correlation coefficient between EZP calculated by Method 1 as a norm and other methods were as follows (Table 7): 0.976 ($p = 0.0009$) for Method 2; 0.968 ($p = 0.002$) for Method 3; 0.962 ($p = 0.002$) for Method 4; 0.608 ($p = 0.20$) for Method 5; and 0.974 ($p = 0.001$) for Method 6.

Table 8 shows the plasma Zn turnover rate, i.e., the sum of flux from the central compartment. Since the turnover rate is determined by the initial slope and the intercept, the turnover rate

determined from the three different models (open three-pool, closed three-pool and the constrained open three-pool models) agreed very well. The correlation coefficient between the TR estimated from the initial two points (5 and 15 minutes) and the TR obtained from the open three-pool (as a norm) was 0.996 ($p = 0.00003$). The percent deviation for the estimate by the initial two points was -4.5 ± 2.4 % (Mean ± SD).

Discussion

There are some limitations and demands of a kinetic study of zinc similar to other nutrients. 1: Chemical analysis of stable isotopes limits the observation period of tracer. 2: Frequency of blood sampling and observation period are limited by experimental design and convenience to the subjects. 3: The 'true' model of zinc kinetics is not established. 4: The model derived from the shorter observation period should be concordant with the model built based on the longer observation period.

Even in plasma or serum, Zn distributes in several compartments biochemically or chemically defined [28]. Tissue Zn is likely distributed in several compartments rather than a single compartment. Most models assume that plasma Zn is in a single compartment [8, 9, 11, 17]. When the number of subcompartments of plasma Zn derived from different chemical species and chemical equilibria among subcompartments are established, the analytical method of the plasma Zn disappearance data must be revised in the future. Some sophisticated multiple compartment modelings utilize tracer data obtained from excreta (urine and feces) and extracorporal detection (liver and thigh) without any knowledge of the chemical speciation.

Based on Miller's model [11] and the analysis of Wastney's data [9], we have chosen a tri-exponential function to fit the disappearance curve of ^{67}Zn from plasma. As a possible three-pool model with all parameters uniquely determined, the mammillary and catenary models with a single outlet from the central compartment were considered. When the discussion is limited to the central compartment (plasma Zn compartment), EZP and TR, the mathematical analysis revealed that the type of model does not affect the results of parameter estimates. Until we will have the 'true' model or the appropriate approximation for the multi-compartment system, invariant parameters will avoid model-based biased comparisons.

It is impractical and unethical to check the adaptability of models using human subjects. We have rather chosen Monte Carlo simulation using Miller's model as the gold standard. Normal random errors were added using the 'Data' procedure of SYSTAT 5. Monte Carlo simulation revealed that the 2% random error is acceptable to estimate the coefficients in the tri-exponential model (Table 3). Therefore, routine ICP-MS analyses that produce measurement errors less than 1 % are suitable for Zn kinetic study.

The analysis of Wastney's data [9] and our data have shown the appropriate number of terms for various observation periods (Table 4) that allows concordance of the shorter observation period to the longer observation period. Therefore, we investigated further the method for

elucidating the practical indicators of the kinetic parameters determined from data based on many time points over 9 days.

During isotopic quasi-equilibrium, the ratio of isotope enrichment in the peripheral compartment to the central compartment is not monotonous and reaches a maximum at the specified time (Appendix 2). Therefore, we selected the time when the ratio reaches a maximum as a time of quasi-equilibrium, because it is uniquely determined (Table 2-1 in Appendix 2; Table 6).

As is shown in Table 5, the open mammillary system derived from 9 day data of ^{67}Zn disappearance from plasma reached 95 % of the maximum ratio of isotope enrichment in pool 1+2+3 (EZP) to pool 1 (plasma Zn or central compartment) 1.0 to 1.3 day after tracer administration, with an average of 1.1 day. These results guarantee that the method estimating EZP and TR developed by the analysis of Miller's mammillary model is valid. We suspect that the 'natural break point' at 2 days after iv dose of tracer proposed by Miller et al [11] may be a literal description of the quasi-equilibrium later than 1 day.

The overestimation from various methods compared to the sum of pools turning over within 48 hours (i.e., the sum of pools 1, 2 and 3 for the open three-pool model) is originated from the quasi-equilibrium and the loss of tracer from the system (Appendix 2; Table 7), as was described by Miller et al [11]. Fortunately, the effect of the quasi-equilibrium is relatively small for estimation of EZP using one-day spot plasma pool because the IER_{1+2+3} / IER_1 at t = 1 day was close to 1 for the subjects. The average loss of the tracer from the system was about 19.7 %, that was similar to the overestimation of EZP by Method 6 (18.7 %). Therefore, the one-day spot plasma pool is a good and practical indicator although its basis is just a single time point.

As a definition, the turnover rate is a product of the initial slope of the time vs semilogarithmic plot of the isotope enrichment and the plasma Zn pool size calculated from the extrapolated intercept to t = 0. The contribution of the later time points is considered smaller than the initial points. The comparison among TR estimated from various methods revealed that the initial two points are practical enough to estimate TR (Table 8).

In conclusion, EZP and TR derived from the open three-pool model using 9-day data are invariant to the models (mammillary or catenary) and can be utilized as a norm for comparison among individual Zn kinetic parameters. Using the closed three-pool model for 1

day, concordant parameters to the longer observation period (9 days) are obtained. One-day spot plasma pool is a practical indicator of EZP. TR can be practically estimated from the initial two points (5 and 15 minutes) instead of a one-day or nine-day observation period.

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Table 1. Characteristics of the subjects

Subject	Sex	Age	Body height	Body weight	Body mass index
		years	m	kg	kg/m^2
1	Male	44	1.80	77.2	23.8
2	Male	65	1.73	93.2	31.2
3	Male	34	1.69	70.2	24.5
4	Male	42	1.73	78.4	26.3
5	Male	24	1.84	83.5	24.6
6	Female	24	1.76	71.9	23.3

Table 2. Parameter estimates of Miller et al's illustration. The remaining tracer in plasma compartment from 5 min - 24 h was calculated from Miller et al's model [11]. The parameters were estimated from the 5 min - 24 h values using the bi-exponential function with a constant term are as follows:

Parameter	Model value	Estimated parameters from the truncated exponential function	Asymptotic standard errors
K_1	0.9545	0.9538	0.0020
g_1	137.6	137.4	0.2
K_2	0.03046	0.03207	0.00006
g_2	3.564	3.322	0.0185
K_3	0.01443	0.01326	0.00004

Where, g_3=0.1106, K_4=0.000628, g_4=0.00232.

These parameters were beyond estimation.

Tracer in plasma in Miller' s model = $K_1e^{-g_1t} + K_2e^{-g_2t} + K_3e^{-g_3t} + K_4e^{-g_4t}$

Tracer in plasma in the truncated exponential = $K_1e^{-g_1t} + K_2e^{-g_2t} + K_3$

Table 3. Mean and SD of the estimated parameters from the Monte Carlo simulation with 100 trials using Miller et al's model. Estimation was based on the values from 5 min-24 h theoretical values given 1 or 2% noise (constant CV) using normal random numbers. SD is the standard deviation of the estimates. CV gives the relative standard error of the estimates.

	1% Random error given			2% Random error given		
Parameter	Mean	SD	CV	Mean	SD	CV
K_1	0.9523	0.0119	1.25%	0.9510	0.0237	2.49%
g_1	137.2	1.2	0.87%	137.1	2.5	1.82%
K_2	0.03201	0.00037	1.16%	0.03196	0.00074	2.32%
g_2	3.318	0.105	3.16%	3.314	0.210	6.34%
K_3	0.01326	0.00024	1.81%	0.01325	0.00049	3.70%

Table 4. Parameter estimates from various observation periods obtained from the analysis of Wastney et al's data [9]

Parameter	0 - 290 days	0 - 28 days	0 - 2 days
K_1	1.18	1.18	1.17
g_1	131	131	130
K_2	0.0433	0.0427	0.0451
g_2	4.50	4.66	4.19
K_3	0.0136	0.0143	0.0136
g_3	0.104	0.118	
K_4	0.00215	0.00228	
g_4	0.00439		

Table 5. Determined coefficients for the tri-exponential model and the truncated model (bi-exponential function with a constant) and the percent deviations of the coefficients of the truncated model from the tri-exponential model

Subject	R^2	K_1	g_1	K_2	g_2	K_3	g_3
Tri-exponential model							
1	0.996	8.80	101.4	0.5753	3.813	0.1912	0.1198
2	0.997	9.25	129.4	0.3238	2.953	0.2813	0.1383
3	0.996	12.04	105.5	0.6164	3.587	0.2893	0.1232
4	0.998	9.16	125.3	0.3449	3.530	0.1834	0.1100
5	0.996	10.98	145.1	0.4239	3.919	0.2387	0.1282
6	0.996	13.82	160.5	0.4049	2.825	0.2122	0.1029
Truncated model							
1	0.998	9.05	106.8	0.6328	5.260	0.2100	n.d.
2	0.996	9.39	132.4	0.3578	3.976	0.2773	n.d.
3	0.996	12.63	114.1	0.7237	6.181	0.3263	n.d.
4	0.997	9.22	126.4	0.3627	3.747	0.1743	n.d.
5	0.996	11.29	149.9	0.4533	5.119	0.2453	n.d
6	0.995	14.27	165.6	0.4190	4.086	0.2329	n.d.
Percent deviation of the truncated model from the tri-exponential model							
1		2.9	5.3	10.0	37.9	9.8	
2		1.5	2.3	10.5	34.6	-1.4	
3		4.9	8.2	17.4	72.3	12.8	
4		0.7	0.9	5.2	6.1	-5.0	
5		2.8	3.3	6.9	30.6	2.8	
6		3.3	3.2	3.5	44.6	9.8	

n.d. not defined.

Table 6. Indicators describing quasi-equilibrium in the open mammillary system found in the subjects

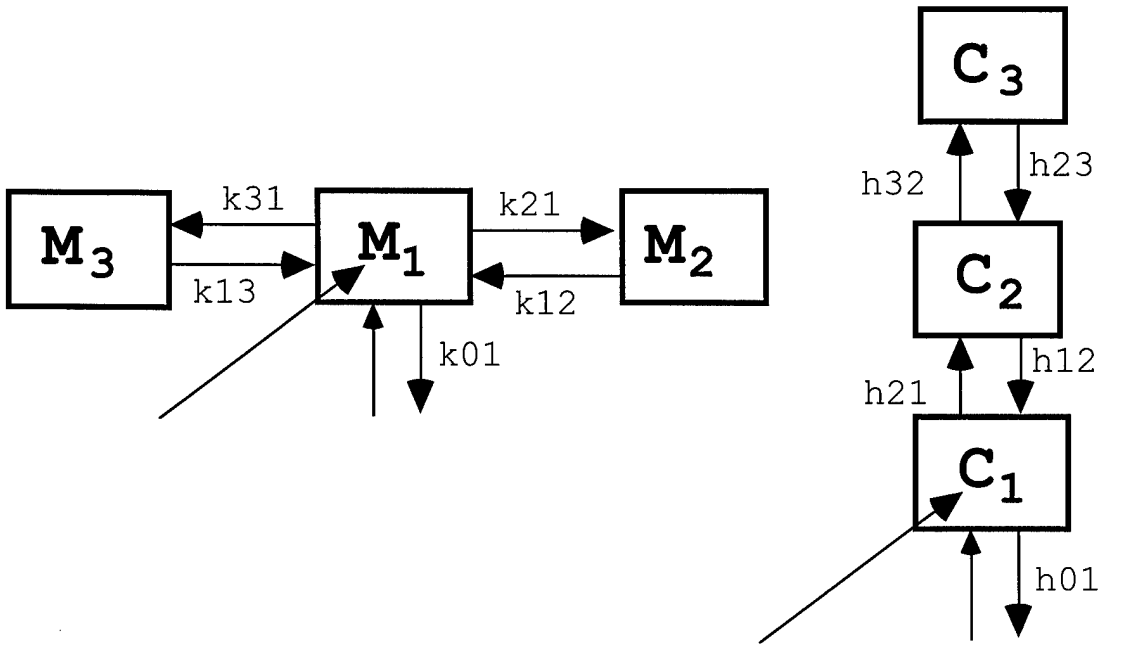
Subject	Time when $IER_{1+2+3} = IER_1$	Time when IER_{1+2+3}/IER_1 takes maximum	Maximum of IER_{1+2+3}/IER_1	Time when IER_{1+2+3} / IER_1 takes 95 % maximum	IER_{1+2+3} / IER_1 at t = 1 day	Remaining tracer within pools 1+2+3 at 1 day later
	day	day		day		%
1	0.810	6.9	1.146	1.085	1.068	77.4
2	0.657	8.2	1.176	1.141	1.094	82.3
3	0.823	6.1	1.119	1.058	1.051	79.0
4	0.844	4.6	1.101	1.035	1.040	81.4
5	0.757	6.9	1.097	1.091	1.056	80.2
6	1.035	7.8	1.109	1.304	0.990	81.6
Mean	0.821	6.8	1.125	1.119	1.050	80.3
SD	0.124	1.3	0.031	0.097	0.035	1.8

Table 7. Comparison of rapidly exchanging Zn pool (EZP) by various methods (mg)

	Method 1	Method 2	Method 3	Method 4	Method 5	Method 6
Subject	Open three-pool*	Closed three-pool ($g_3 = 0$)	Constrained open three-pool ($g_3 = 0.120$)	Last term of tri-exponential	Simple extra-polation	One day spot plasma pool
	5 min - 9 d	5 min - 1 d	5 min - 1 d	5 min - 9 d	3 - 9 d	1 d
1	169	202	148	222	281	206
2	143	172	133	169	190	175
3	131	146	113	164	214	153
4	191	244	178	232	242	233
5	166	194	151	200	233	195
6	162	182	137	200	218	182
Percent deviation from Method 1						
1		19.5	-12.3	31.4	66.3	21.9
2		20.0	-7.2	18.3	33.1	22.1
3		11.3	-13.8	25.5	63.0	16.7
4		27.7	-6.9	21.5	26.7	22.0
5		16.9	-8.8	20.5	40.4	17.5
6		12.3	-15.5	23.5	34.6	12.3

Table 8. Comparison of plasma Zn turnover rate (TR) determined from various models (mg/day)

Models	Open three-pool	Closed three-pool ($g_3 = 0$)	Constrained open three-pool ($g_3 = 0.120$)	Initial two points (5 and 15 minutes)
Interval	5 min - 9 d	5 min - 1 d	5 min - 1 d	5 and 15 min
1 male	415	421	415	406
2 male	587	589	592	557
3 male	361	367	361	358
4 male	520	521	520	488
5 male	561	562	559	528
6 female	452	450	451	421
Mean	483	485	483	460
SD	88	86	89	77
CV	18	18	18	17



Mammillary Model **Catenary Model**

Figure 1. Mammillary and catenary models with three pools

M_1 corresponds to the central compartment in the mammillary model.
C_1 corresponds to the central compartment in the catenary model.
M_2 and M_3 are the peripheral pools.
C_2 and C_3 are the peripheral pools.

Mammillary model is a linear kinetic system which has noncentral or peripheral pools, each separately connected to a central pool without interconnection among peripheral pools.

Catenary model is a linear kinetic system which has several pools sequentially connected each other in the chain form.

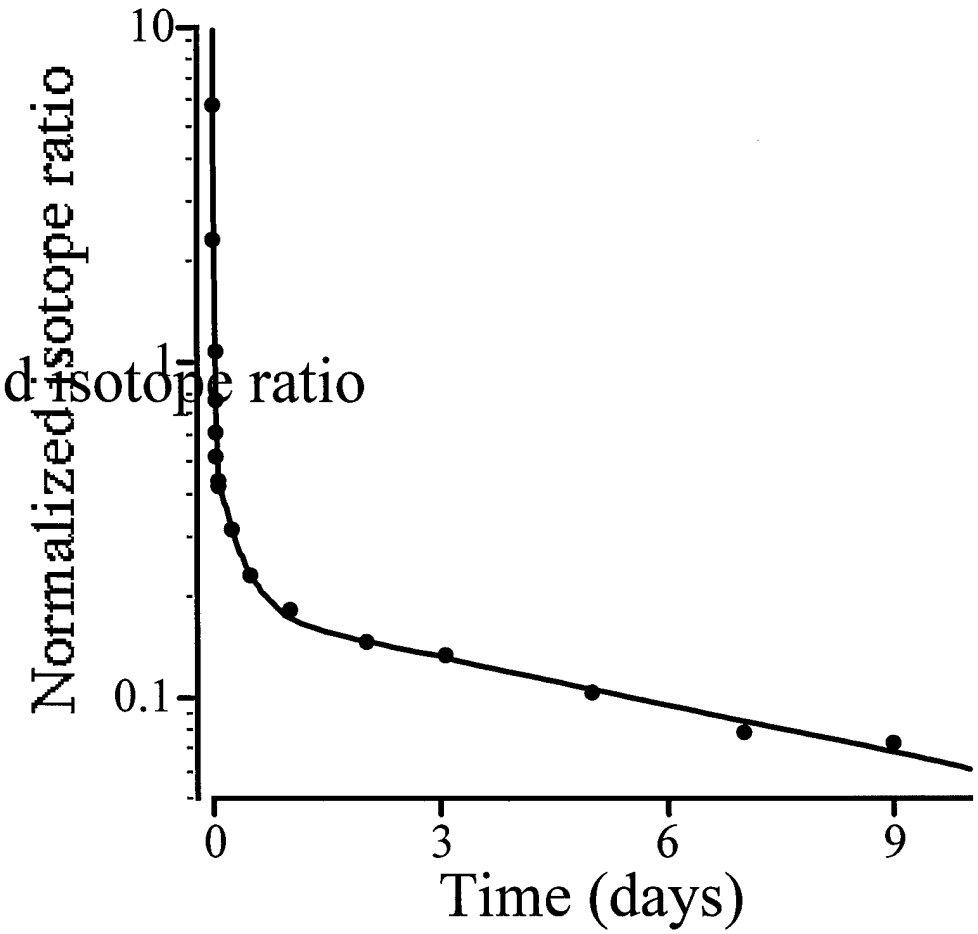


Figure 2. Illustration of the disappearance curve fitted to a triexponential function (Data were obtained from Subject NE). Simplex minimization of residual square by nonlinear regression of SYSTAT Software using the following model equation:
Logarithm of normalized isotope ratio = $Log\left(K_1 e^{-g_1 t} + K_2 e^{-g_2 t} + K_3 e^{-g_3 t}\right.$

Appendix 1.

Solving the mammillary model

Using Landaw et al's [24] algorithm

$$k_{11} = k_{01} + k_{21} + k_{31} = \frac{\sum_{i=1}^{3} K_i g_i}{\sum_{i=1}^{3} K_i}$$

Roots of the numerator after Laplace transformation of tri-exponential function give: $k_{22} = k_{12} + k_{21}$ and $k_{33} = k_{13} + k_{31}$.

$$\gamma_j = k_{ij} k_{ji} = \frac{\sum_{i=1}^{3} K_i}{\sum_{i=1}^{3} \frac{K_i}{\left(k_{jj} - g_i\right)^2}}$$

For the single outlet or closed model, Q_2, Q_3, and 'k's are uniquely determined:

$$k_{22} = k_{12}$$

$$k_{33} = k_{13}$$

$$Q_1 = \text{Dose of tracer (mmol)} \Big/ \left(K_1 + K_2 + K_3\right)$$

$$Q_2 = \gamma_2 / k_{12}\ Q_1$$

$$Q_3 = \gamma_3 / k_{13}\ Q_1$$

$$\text{Rapidly Exchangeable Zn Pool (EZP)} = Q_1 + Q_2 + Q_3$$

where Q1 is the plasma Zn compartment (central compartment).

Appendix 2
Quasi-equilibrium in Miller's mammillary model

Tracer / tracee ratios in the respective compartments in Miller's mammillary model can be solved by both the numerical and analytical methods.

Numerical solution of Miller's Mammillary model
The following is the "Mathematica" statement that describes the numerical solution of Miller's mammillary model (open four-pool/single outlet).

```
NDSolve[{qa'[t] == 0.92 qc[t] + 0.0064 qd[t] + 8.9 qb[t]
- (2.4 + 40 + 4 + 85) qa[t],
qb'[t] == 85 qa[t] - 8.9 qb[t],
qc'[t] == 40 qa[t] - 0.92 qc[t],
qd'[t] == 4 qa[t] - 0.0064 qd[t],
qa[0] == 1, qb[0] == 0, qc[0] == 0, qd[0] == 0},
{qa, qb, qc, qd}, {t,0.001,10}]
```

where q_a [t], q_b [t], q_c [t] and q_d [t] correspond to the amount of tracer at time t in the respective pools (a, b, c and d) in Figure 2-1.

Analytical solution of Miller's mammillary model

$$q_a(t) = 0.95449e^{-137.55t} + 0.030457e^{-3.56365t} + 0.0144254e^{-0.110596t} + 0.000628113e^{-0.00231993t}$$

$$q_b(t) = -0.630638e^{-137.55t} + 0.485133e^{-3.56365t} + 0.139504e^{-0.110596t} + 0.0060004e^{-0.00231993t}$$

$$q_c(t) = -0.279438e^{-137.55t} + 0.460832e^{-3.56365t} + 0.712891e^{-0.110596t} + 0.0273783e^{-0.00231993t}$$

$$q_d(t) = -0.0277582e^{-137.55t} + 0.0342478e^{-3.56365t} + 0.55378e^{-0.110596t} + 0.615786e^{-0.00231993t}$$

$$IER_a(t) = q_a(t)/Q_a = q_a(t)/0.037$$

$$IER_b(t) = q_b(t)/Q_b = q_b(t)/0.35$$

$$IER_c(t) = q_c(t)/Q_c = q_c(t)/1.6$$

$$IER_d(t) = q_d(t)/Q_d = q_d(t)/23$$

where q_a [t], q_b [t], q_c [t] and q_d [t] correspond to the amount of tracer at time t in the respective pools (a, b, c and d); IER_a, IER_b, IER_c and IER_d are the isotopic enrichment in pools a, b, c and d in Figure 2-1.

'The results are shown in Figure 2-2 and 2-3.

Figure 2-2 and 2-3, and Table 2-1 show the result of the calculation regarding the quasi-equlibrium in Miller's mammillary model.

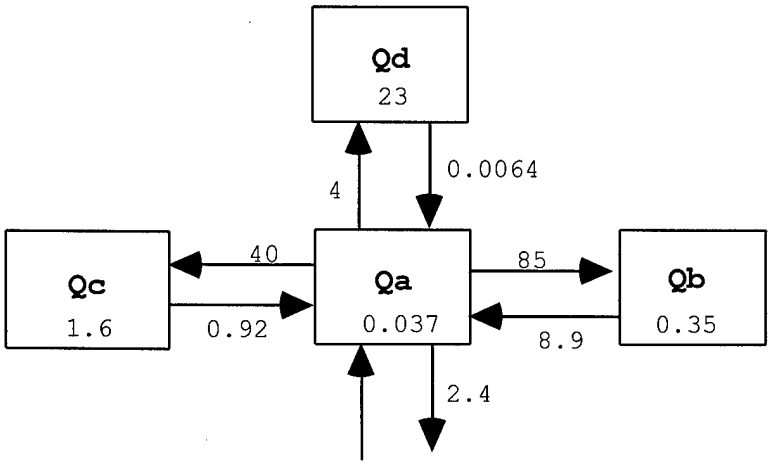
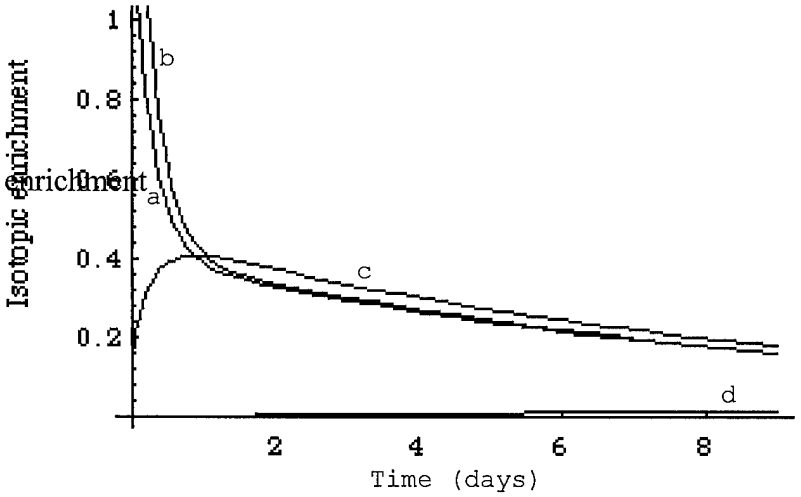


Figure 2-1. Miller's mammillary model
The unit for pool sizes are mmol. The unit for the rate constant is day^{-1}.



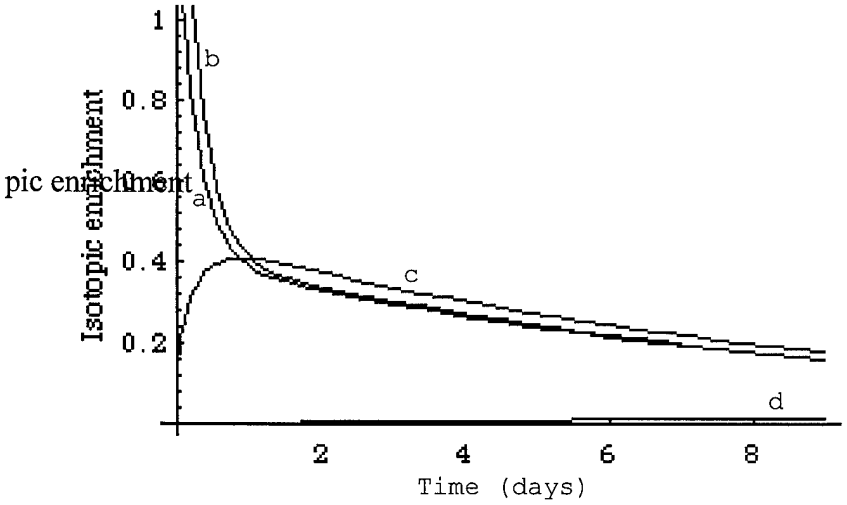


Figure 2-2. Isotopic enrichment in the compartment of Miller's mammillary model

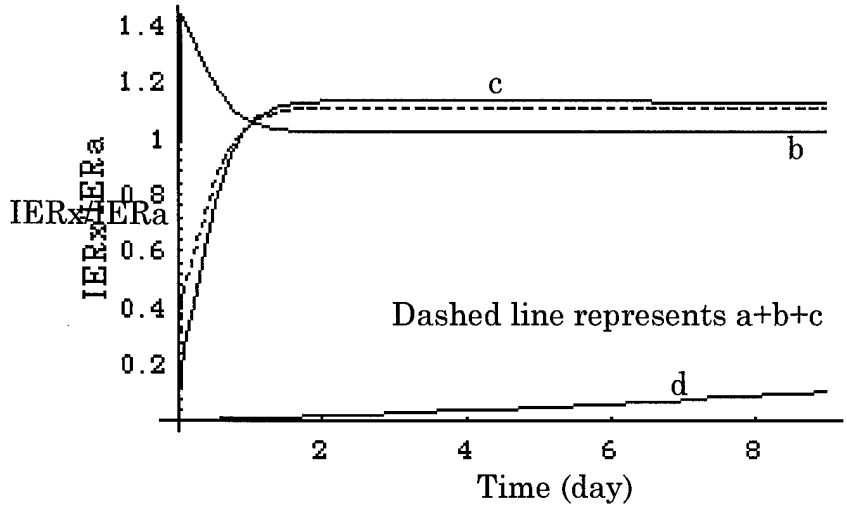


Figure 2-3. The ratio of isotopic enrichment in pool x to pool a (IER_x/IER_a).

x represents b, c and a+b+c. a+b+c represents the composite of pools a, b and c. About one day after the intravenous administration of tracer, pool b and the composite of pools a, b and c reach 95 % of maximum and are in the state of the quasi-equilibrium. Overshoot was observed in IER_b/IER_a.

Table 2-1. Indicators describing quasi-equilibrium in Miller's mammillary model

Pool	Time when $IER_x = IER_a$	Time when IER_x / IER_a takes maximum	Maximum of IER_x / IER_a	Time when IER_x / IER_a takes 95 % maximum	IER_x / IER_a at t = 1 day
	day	day		day	
b	0.029	0.066	1.426	0.200	1.061
c	0.866	2.752	1.135	1.130	1.047
d	34.575	718.608	1.577	56.512	0.013
a+b+c	0.822	2.718	1.113	1.040	1.049

[1] a,b,c and d represent pools in Miller's mammillary model.

[2] a+b+c represents composite of pools a, b and c.

[3] x represents a, b, c or d.

[4] IER represents isotopic enrichments.

Appendix 3.

Conserved parameters by matrix transformation of three pool systems of mammillary and catenary models

When the disappearance curve is described by the tri-exponential function:

$$q_1(t) = K_1 e^{-g_1 t} + K_2 e^{-g_2 t} + K_3 e^{-g_3 t}$$

where $q_1(t)$ is enrichment in the central Zn compartment, 'K's are linear coefficients, 'g's are exponential coefficient and t is time in days. To give the order to the terms, $g_1 > g_2 > g_3$ is defined without loosing the generality.

Since the pool size of the central compartment is given by the iv dose (D, mmol) divided by the extrapolated enrichment.

$$q_1(0) = K_1 + K_2 + K_3 \quad : Extrapolated enrichment\ at\ t = 0.$$

$$M_1 = \frac{D}{q_1(0)}$$

$$C_1 = \frac{D}{q_1(0)}$$

$$\therefore M_1 = C_1$$

where D is the intravenous dose of the tracer.

The sum of the rate constants from the central compartments is the initial slope of the disappearance curve divided by $q_1(0)$.

$$k_{11} = k_{01} + k_{21} + k_{31}$$

$$= \frac{\sum_{i=1}^{3} (K_i g_i)}{\sum_{i=1}^{3} (K_i)}$$

$$h_{11} = h_{01} + h_{21}$$

$$= \frac{\sum_{i=1}^{3} (K_i g_i)}{\sum_{i=1}^{3} (K_i)}$$

$$\therefore \quad k_{11} = h_{11}$$

$$F_{m\,11} = k_{11} M_1$$

$$F_{c\,11} = h_{11} C_1$$

$$\therefore \quad F_{m\,11} = F_{c\,11}$$

where F_{m11} and $F_{c11}$is the flux from the central compartment of the mammillary model and the catenary model, respectively.

Ramakrishnan (1984) reported the 'indistinguishability' between the mammillary and catenary models and reported the matrix transformation.

For the mammillary model,

$$A = \begin{bmatrix} -k_{11} & k_{21} & k_{31} \\ k_{12} & -k_{22} & 0 \\ k_{13} & 0 & -k_{33} \end{bmatrix} = \begin{bmatrix} -(k_{01}+k_{21}+k_{31}) & k_{21} & k_{31} \\ k_{12} & -k_{12} & 0 \\ k_{13} & 0 & -k_{13} \end{bmatrix}$$

$$q_m(0) = (q_1(0) \quad 0 \quad 0)$$

$$\frac{dq_m}{dt} = q_m A$$

At the steady state,

$$\frac{dM_1}{dt} = F_{m10} + k_{12}M_2 + k_{13}M_3 - k_{11}M_1 = 0$$

$$\frac{dM_2}{dt} = k_{21}M_1 - k_{12}M_2 = 0$$

$$\frac{dM_3}{dt} = k_{31}M_1 - k_{13}M_3 = 0$$

$$M_2 = \frac{k_{21}M_1}{k_{12}}$$

$$M_3 = \frac{k_{31}M_1}{k_{13}}$$

$$M_1 + M_2 + M_3 = \left(1 + \frac{k_{21}}{k_{12}} + \frac{k_{31}}{k_{13}}\right)M_1$$

For the catenary model transformed from the mammillary model,

$$B = \begin{bmatrix} -h_{11} & h_{21} & 0 \\ h_{12} & -h_{22} & h_{32} \\ 0 & h_{23} & -h_{33} \end{bmatrix} = \begin{bmatrix} -h_{11} & h_{21} & 0 \\ h_{12} & -(h_{12}+h_{32}) & h_{32} \\ 0 & h_{23} & -h_{23} \end{bmatrix}$$

$$q_c(0) = \begin{pmatrix} q_1(0) & 0 & 0 \end{pmatrix}$$

$$\frac{dq_c}{dt} = q_c\, B$$

$$= q_c\, T^{-1} A T$$

$$Select\ T^{-1} = P = \begin{bmatrix} 1 & 0 & 0 \\ 0 & \dfrac{k_{21}}{k_{31}+k_{21}} & \dfrac{k_{31}}{k_{31}+k_{21}} \\ 0 & \dfrac{k_{13}}{k_{13}-k_{12}} & \dfrac{k_{12}}{k_{12}-k_{13}} \end{bmatrix}$$

$$\frac{dq_c}{dt} = q_c\, PAP^{-1}$$

Matrix transformation from the mammillary model to the catenary model is as follows: (Remark. In his PAP^{-1} matrix (p. 382), "-" was deleted (typographically?) from the 3, 3rd entry.)

$$B = PAP^{-1}$$

$$= \begin{bmatrix} -k_{11} & k_{31}+k_{21} & 0 \\ \dfrac{k_{31}k_{13}+k_{21}k_{12}}{k_{31}+k_{21}} & -\dfrac{\left(k_{31}k_{13}^2+k_{21}k_{12}^2\right)}{k_{31}k_{13}+k_{21}k_{12}} & \dfrac{k_{21}k_{31}\left(k_{12}-k_{13}\right)^2}{\left(k_{21}+k_{31}\right)\left(k_{31}k_{13}+k_{21}k_{12}\right)} \\ 0 & \dfrac{k_{12}k_{13}\left(k_{31}+k_{21}\right)}{k_{31}k_{13}+k_{21}k_{12}} & -\dfrac{k_{12}k_{13}\left(k_{31}+k_{21}\right)}{k_{31}k_{13}+k_{21}k_{12}} \end{bmatrix}$$

At the steady state,

$$\frac{dC_1}{dt} = {}_cF_{10} + h_{12}C_2 - h_{11}C_1 = 0$$
$$\frac{dC_2}{dt} = h_{21}C_1 + h_{23}C_3 - \left(h_{12}+h_{32}\right)C_2 = 0$$
$$\frac{dC_3}{dt} = k_{32}C_2 - k_{23}C_3 = 0$$

$$C_2 = \frac{h_{21}C_1}{h_{12}}$$
$$= \frac{\left(k_{21}+k_{31}\right)^2}{k_{12}k_{21}+k_{13}k_{31}}M_1$$
$$C_3 = \frac{h_{32}C_2}{h_{23}}$$
$$= \frac{\left(k_{12}-k_{13}\right)^2 k_{21}k_{31}}{k_{12}k_{13}\left(k_{12}k_{21}+k_{13}k_{31}\right)}M_1$$
$$\therefore C_1 + C_2 + C_3 = \left(1+\frac{k_{21}}{k_{12}}+\frac{k_{31}}{k_{13}}\right)M_1$$
$$= M_1 + M_2 + M_3$$

Summary of the conserved parameters

1. Pool size of the central compartment (plasma Zn pool)
2. Sum of the rate constant from the central compartment
3. Flux from the central compartment (plasma Zn turnover rate)
4. Sum of the pool sizes (so-called rapidly exchangeable Zn pool, EZP)

This manuscript will be submitted for publication to Analytica Chimica Acta journal soon.

SIMPLIFIED PRETREATMENT METHOD FOR THE ANALYSIS OF PLASMA SAMPLES APPLICABLE TO ZINC KINETICS AND INDUCTIVELY COUPLED PLASMA - MASS SPECTROMETRY

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ABSTRACT

Common biochemical indicators of zinc, such as plasma Zn are insensitive. Radioisotope, ^{65}Zn, has been used to show the rapid plasma Zn disappearance in Zn deficiency. However, the radiation exposure limits its application. Stable Zn isotopes are alternatives. Sample purification is usually required to obtain accurate results for mass spectrometric analysis, but also increases the chance of contamination. Prior to comparing the isotope ratio (IR) results from "extracted" and "nonextracted" hydrogen peroxide digested samples, the background counts generated from polyatomic interferences at zinc isotopic masses 64, 66, 67, 68, and 70 were established by subjecting a series of various "simulated human plasma" mineral solutions of single and mixtures of double and all possible mineral elements (S, Na, Cl, K, P, and Ca) to IR measurements using the inductively coupled plasma-mass spectrometry (ICP-MS). The mixture of all mineral elements, usually encountered in the digested human plasma, interfered only with ^{64}Zn (6.66 ng/mL) and ^{70}Zn (8.51 ng/mL). However, the interferences to ^{66}Zn, ^{67}Zn, and ^{68}Zn are minimal containing 0.90, 0.94, and 0.39 ng/mL, respectively. The mixture of S - Cl, Na - Cl reduced the interference with ^{67}Zn. These results also suggested that co-presence of Na or S affects the chemical reaction of Cl in argon plasma, and the major interferent is shifted from $^{35}Cl^{16}O_2$ (atomic mass 67 coming from Cl solution) to $^{35}Cl_2$.

For the comparison of two pretreatment methods (extraction vs nonextraction), plasma samples were collected from ten human subjects 5 min to 24 h and four subjects 5 min to 9 days after injecting 2 mg ^{67}Zn intravenously (i.v.). The plasma (1.5 ml) was digested by hydrogen peroxide (Alcock, 1987) and dissolved in nitric acid. **"Extraction":** Zn in the digestate was extracted into CCl_4 as diethylammonium diethyldithiocarbamate chelate followed by back extraction of Zn in nitric acid. The solution was then heated overnight at 80°C to remove traces of CCl_4, and made up to 10 ml with high purity water after adding yttrium (Y) internal standard. **"Nonextraction":** Y and high purity water was directly added to the digestate. After subtraction of the baseline the plasma Zn IR was divided by the natural Zn IR to give the normalized IR (NIR). Logarithmically transformed NIR was calculated for regression analysis. NIRs $^{67/66}Zn$ and $^{67/68}Zn$ obtained from the extracted samples agreed well (r^2= 0.998). The NIRs obtained from $^{67/66}Zn$ and $^{67/68}Zn$ by both the methods agreed well compared to those from other ratios ($^{67/64}Zn$, r^2=0.838; $^{67/66}Zn$, r^2= 0.976; $^{67/68}Zn$, r^2=0.985; $^{67/70}Zn$, r^2=0.747). Considering the minimum possibility of isobaric interferences in plasma samples, $^{67/68}Zn$ obtained from nonextracted samples is sufficient for routine Zn kinetic analysis.

INTRODUCTION

For the assessment of Zn nutriture, determinations of plasma/serum Zn concentrations are used extensively in clinical practice, with normal plasma/serum Zn concentrations generally considered to be 700-1200 ug/L. However, these concentrations are known to be influenced by many physiological factors (Goldenberg et al., 1995). There is a need for improved techniques to assess Zn status and monitor Zn metabolism since the Zn content of accessible tissues does not appear to provide a reliable index of Zn status and plasma or serum zinc concentrations vary with stress conditions unassociated with Zn deficiency. Isotopic techniques seem to provide an answer to this problem.

Radioisotopes of Zn have been used to develop complex mathematical models which describe Zn kinetics under various conditions in man and laboratory animals (Foster et al, 1979; Henkin et al., 1984; Wastney et al, 1986; Dunn and Cousins, 1989). Such models have been used to identify sites of regulation of Zn metabolism and calculate the size and turnover rate of body Zn pools. A simpler model describing Zn kinetics over a short time period (90 minutes) has been developed using ^{65}Zn in the rat (Lowe et al, 1991). Using this model it was shown that a rapidly exchanging pool of

Zn is responsive to changes in dietary Zn intake, becoming significantly depleted in animals maintained on a Zn deficient diet. ^{65}Zn has a biological half-life of 500 days (Hawkins et al., 1976), and hence its applicability in the study of Zn metabolism in animals or humans is limted.

A potential approach to the study of relationships between dietary Zn supply and body status is the measurement of plasma Zn kinetics following an intravenous injection (i.v.) of a nonradioactive stable Zn isotope. Stable isotopes offer an advantage over radioisotopes in that there is no radiation exposure of the subjects. Stable Zn isotopes offer a clear advantage over radioisotopes in that they occur naturally and hence have been used to study various aspects of zinc metabolism in humans (Turnlund et al., 1982, 1984, 1986; Istfan et al., 1983, Miller et al., 1994), although Jackson et al. (1984, 1988) used ^{67}Zn to examine Zn turnover rates in humans. Several instrumental techniques have been reported for the determination of isotope ratios of Zn including neutron activation analysis (Janghorbani et al., 1980; Gokman et al., 1989; Wastney et al., 1986, 1991) and mass spectral methods such as thermal ionization (Fairweather-Tait et al., 1993), fast atom bombardment (Peirce et al., 1987, Friel et al., 1992; Miller et al., 1994; Sian et al., 1996), and inductively coupled plasma (Serfass, 1986, Lowe et al., 1993, Friel et al., 1993)

Recently, we (Yokoi et al., 1994a,b) and others (Jackson et al., 1988; Lowe et al., 1991, 1993) have shown that stable isotopes of Zn can be successfully used to measure Zn turnover rates (TR) and exchangeable Zn pools (EZP) which are responsive to changes in Zn status. The availability of and ability to measure stable isotopes of Zn by mass spectrometry make this a viable technique for Zn metabolic studies. Studies from our laboratory indicate that inductively coupled plasma-mass spectrometry (ICP-MS) provides reliable detection of the isotopes of Zn.

The stable isotope kinetics methodology for the determination of Zn status involves: (i) the use of intravenously administered Zn-67 stable isotope tracer, collection of blood at various time points, and (ii) determination of isotope ratios $^{67/64}Zn$, $^{67/66}Zn$, $^{67/68}Zn$, and $^{67/70}Zn$ at each time point using ICP-MS. The data are used to calculate Zn disappearance and turnover rates, and the exchangeable Zn pools after injection. The findings are related to biochemical and physiological indices of functions in order to establish zinc status.

Extraction of Zn from samples has usually been performed to obtain accurate results for mass spectrometric analysis (Serfass et al., 1986;

Miller et al., 1994; Yokoi et al., 1994 a,b). Unfortunately, the extraction also increases the chances of contamination since Zn is ubiquitous in the environment. The major matrix elements in human blood samples are sodium (Na, 3250 ppm), chloride (Cl^-, 3500 ppm), and sulfur (S, 1200 ppm) (Vanhoe et al., 1989). Theoretically there are no apparent major isobaric interferences for ^{66}Zn and ^{68}Zn in blood plasma, although $^{32}S^{16}O_2$ and $^{32}S_2$ overlap ^{64}Zn. Hence, we wanted to test this theory by conducting a detailed study of comparing the isotope ratios obtained from several sets of "extracted" and "nonextracted" plasma samples. In this project we compared the four different isotope ratios obtained from Zn-extracted and Zn-nonextracted digested plasma samples in order to determine whether the "nonextraction" procedure (with little or no contamination) is applicable for routine Zn isotope ratio analysis for kinetic studies in humans.

EXPERIMENTAL

Chemicals, Reagents and Supplies

The enriched stable isotope ^{67}Zn (as oxide, purity 93.11%) was purchased from Oak Ridge National Laboratory (Martin Marietta Energy Systems, Inc., Oak Ridge, TN, U.S.A.). Hydrogen peroxide (30%, ultrapure), nitric acid (ultrapure double-distilled from Vycor), ammonium hydroxide (high purity grade), hydrochloric acid (ACS grade) and sufuric acid (ACS grade) were purchased from GFS Chemicals, OH, U.S.A. Hydrochloric acid (suprapure grade) was obtained from EM Science, Gibbstown, NJ.

Sodium nitrate, diammonium monohydrogenphosphate and calcium carbonate (Baker analyzed chemical grades) were purchased from Baker, Phillipsburg, NJ. Potassium nitrate (ACS grade) was obtained from Fisher Chemicals. Carbon tetrachloride (ACS grade) and 2,6-dinitrophenol, and Zn and yttrium reference standard solutions were purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Diethylammonium diethyldithiocarbamate was purchased from Tokyo Casei Co., Tokyo, Japan.

Deionized water was prepared using a Milli-Q System (Millipore Corp., Milford, MA, U.S.A.). Monovette syringes containing lithium heparin (10 U/mL blood) used for blood collections and polypropylene sterile tubes (29 x 114 mm size with red caps) free from Zn contamination used for plasma digestions were purchased from Sarstedt Inc., Newton, NC, U.S.A. Disposable Falcon polypropylene tubes (15 mL capacity) used for preparing the final ICP-MS digestate solutions and absolute ethanol used to dissolve the 2,6-dinitrophenol indicator were purchased from Fisher Scientific Co., Pittsburgh, PA, U.S.A. The carbon tetrachloride extraction of Zn from the

digestates were carried out in hydrochloric acid (10%) washed borosilicate glass tubes (Kimax Inc., Toledo, OH, U.S.A.).

Human Subjects and Zinc Kinetics

Five healthy men and one woman living in Galveston, Texas were the subjects for the 9-day observation. Eleven women in apparent good health who were participating in a study of effects of zinc and iron status on brain function participated in 24-hour observation study. This project was approved by the Institutional Review Board of the University of Texas Medical Branch and written consent was obtained from all subjects. The disappearance rate for ^{67}Zn from blood plasma, turnover rate, and the exchangeable Zn pool sizes were measured using the procedures well established in our laboratory (Yokoi et al., 1994 a,b; Yokoi et al., 1997; Sadagopa Ramanujam et al., 1997; Egger et al., 1997).

Zinc kinetics were measured using ^{67}Zn (natural abundance 4.11%; enrichment, 93.11%) chloride which was prepared from ^{67}Zn oxide by dissolving 59.52 mg in a few drops of concentrated hydrochloric acid (ACS grade, GFS Chemicals, Columbus, OH), and heating it to dryness on a hot plate. The synthesized chloride was dissolved in saline (12 mL, corresponds to 0.5 mL = 2 mg of ^{67}Zn), aliquots of 0.5 mL sterilized by passing the solution through Millipore filter (0.2 uM pore size) into glass vials containing 10.0 mL saline. Several of these vials (one per 10 vials) were randomly selected and tested for sterility (University of Texas Medical Branch, Department of Clinical Microbiology and Immunology) and pyrogenicity (Scientific Associates Inc., St. Louis, MO).

Subjects were admitted to the Clinical Research Center for administration of ^{67}Zn and collection of blood samples. The diet was limited in bioavailable zinc. At 07:00 A.M., after the subject had fasted at least 12 hours, short Teflon catheters connected to a 3-way stop cock were placed in each anticubital vein and kept open by 0.9% saline solution. After 30 min, a blood sample was taken to establish the baseline $^{67/68}Zn$ ratio.

Then the ^{67}Zn tracer - 2 mg in 10 mL saline further diluted to 30 mL in saline - was administered over 3 min (timed by stop watch). The line was flushed rapidly with saline for 30 seconds. Blood samples were then collected from the opposite arm starting 5 min after completion of the

^{67}Zn administration. Additional samples were collected at 5, 15, 30, 40, 50, 60, 90 minutes, and 2, 6, 12 hours, and 1, 2, 3, 5, 7, and 9 days later. The 9-day and 1-day samples were collected from 4 and 10 subjects, respectively. Blood samples were placed in an ice chest after collection and delivered to the laboratory for processing. The blood samples were centrifuged at 2000 rpm for 20 minutes and the plasma layers transferred to polypropylene tubes and stored in a freezer (-70 C) until ready to use.

Digestion of Plasma and Extraction of Zinc

Sample digestion was based on the method of Alcock (1987). Duplicate aliquots of plasma were measured out in 50 mL polypropylene tubes, kept overnight at -70°C, transferred to a freeze-drier and lyophilized overnight, further dried for 8 hours at 80°C in an oven, and digested with 30% hydrogen peroxide (2 aliquots of 5 and 7 mL, high purity grade, GFS Chemical Co.) for 2 days at 85-90°C. The white ash was dissolved in 1.5 mL 1.2N Ultrapure nitric acid. Several (4 tubes/batch) hydrogen peroxide blanks run throughout the entire procedure were used to calculate ICP-MS IR blank subtractions.

"Extraction": The extraction of Zn was based on the method of Serfass et al (1986) modified by Yokoi et al (1994). After digestion of the plasma, the ash was dissolved in 1.5 mL of 1.2 M nitric acid and the solution transferred to 20 mL acid-washed borosilicate tube using the Zn-free polyethylene transfer pipette followed by two washes with deionized water. One drop (40 uL) of 0.1% 2,6-dinitropehenol in 50% ethanol was added to the solution as a pH indicator. Dilute ammonium hydroxide was added in drops with shaking the tube to bring the pH to 2.5 (indicated by the color change to yellow). One mL of 0.25% diethylammonium diethyldithiocarbamate in carbon tetrachloride was added, the tube closed tight with Teflon-lined cap, and the contents shaken vigorously for 2 minutes. Each tube was allowed to stand until separation of the acid and carbon tetrachloride layers was complete.

The carbon tetrachloride layer containing chelated zinc was transferred to another glass tube carefully using the acid-washed glass pasteur pipette, the Zn-chelate decomposed with 1 mL of 1.2 M nitric acid, and the Zn back-extracted into the acid by vigorously shaking the tube. The back-extration of Zn was usually indicated by the transfer of yellow color into the acid layer followed by its disappearance. If such a transfer did not occur immediately, the solution was allowed to stand for an hour and shaken again to complete the decomposition and transfer steps. Then the top acid layer was transferred to another glass tube and the solution heated overnight at 80^{o}C to remove traces of CCl_4, and made up to 10 ml

with Milli-Q deionized water after adding yttrium internal standard (100 uL of 5 mg/L solution in 1% nitric acid). Batches of 12-20 tubes were processed at one time.

"Nonextraction": After digestion of the plasma, the ash was dissolved in 1.5 mL of 1.2 M nitric acid and each solution transferred to a polypropylene falcon tube followed by two washes with Milli-Q deionized water. The Yttrium internal standard and Milli-Q water were directly added to the digestate and made up to 10 mL with water.

Solutions for the Measurement of ICP-MS Interferents

Because the preparation of human plasma for isotope ratio ICP-MS analysis involves digestion by hydrogen peroxide and solubilization of the obtained white ash with nitric acid, the main matrix elements in the well digested solution should only be hydrogen, oxygen and nitrogen. The polyatomics that interfere with zinc isotopes (64, 66, 67, 68 and 70 atomic mass units) are limited to the combination of the main matrix (H, O and N), the plasma minerals (Na, Cl, S, K, P and Ca) and argon (Ar, used as a source for the inductively coupled plasma). Therefore the prepared mineral solutions contained only hydrogen, oxygen and nitrogen except for the focused mineral elements (Na, Cl, S, K, P and Ca) to satisfy the above requirements.

Single mineral solutions that contained the respective mineral found in the "actual human plasma" were prepared by dissolving each salt or diluting each acid in Milli-Q water. Nitric acid (1.2 N) was prepared by diluting the concentrated nitric acid using Milli-Q water. Calcium nitrate prepared from calcium carbonate was used to make the calcium solution. Calcium carbonate (1250 mg) was dissolved by a few drops of nitric acid and the excess acid was evaporated by heating on a hot plate to obtain calcium nitrate. To start with, 3600 μg Na/ml as sodium nitrate, 3300 μg Cl/ml as hydrochloric acid, 1200 μg S/ml as sufuric acid, 189 μg K/ml as potassium nitrate, 141 μg P/ml as diammonium monohydrogenphosphate, and 99 μg Ca/ml as calcium nitrate were prepared. At the second step, the solutions containing one tenth of each mineral concentration that usually found in the representative human plasma were prepared in 0.12 M nitric acid with 50 ng Y/ml as an internal standard. Single mineral solutions contained only one mineral element from Na, Cl, S, K, P and Ca. The mixture of two minerals contained two different kinds of mineral elements. The mixture of all minerals contained all the 6 mineral elements (Na, Cl, S, K, P and Ca). In addition, the mixture of S, Na and Cl was made to test the "piling up effect" of the interferents or polyatomics.

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

A VG PlasmaQuad-1, upgraded to PlasmaQuad-2 plus status (VG Instruments, Winsford, England, U.K.) ICP-MS instrument was used for all isotope ratio measurements. Each solution was aspirated and nebulized (Meinhardt concentric type) into the argon plasma (8000-6000^{o} K) via a peristaltic pump with a flow rate of approximately 1 mL/min. The yttrium (mass 89) internal standard was used to correct errors due to instrumental drifts during data acquisitions. Isotope ratio analyses were performed using "Peak-Jump Acquire" Isotope Ratio data acquisition mode of the PlasmaQuad software. The peak-jump acquisition mode gave better relative standard deviations (RSD <1%) compared to the scan acquisition mode (2-4%). The mass range scanned was 50-95 amu with 200 scan sweeps of 2048 channels, 160 microsec dwell time per channel, and 200 peak jump sweeps with 10240 microsec per peak jump sweep. These mass spectral acquisition parameters normally require about 9 mL of solution and 25 minutes acquisition time for ten replicate measurements of each sample. Instrument control, methods procedures and the data system, including calculations and statistics, were operated via a Compaq AT personal computer with version 3.2 of the V.G. PlasmaQuad software . All the Four Zn isotope ratios ($^{67/64}$Zn, $^{67/66}$Zn, $^{67/68}$Zn, $^{67/70}$Zn) were measured in each sample. The mass discrimination among Zn isotopes was corrected by the frequent measurements of Zn standard solutions (125, 250, and 500 ng/mL) during the sequence of IR analysis.

Measurement of Polyatomic Interferents in the "Simulated Human Plasma" Solutions

After careful cleaning of the sampling/skimmer cones, torch, nebulizer, and the spray chamber, the various "simulated human plasma" mineral solutions prepared to quantify the ICP-MS interferents were introduced into the argon plasma and the counts at the desired atomic mass units (64, 66, 67, 68, 70 and 89) were recorded using "Peak-Jump Acquire" Isotope Ratio data acquisition mode as described earlier. The counts obtained at the desired atomic mass units were compared with the counts obtained from 250 ng Zn/ml and the equivalent concentrations of the interferents were calculated.

In the analysis of the digested human plasma without any purification process, we always observed the "piling up effect" in the counts at 64 atomic mass units. To test "piling up effect", 72 (for S) and 36 (for S - Na and S - Cl) consecutive runs were used. In addition, 48 consecutive runs were utilized to see complete washing out of the interferents. In the first 36 runs, the mineral solution containing S, Na

and Cl were introduced into the argon plasma. Right after the 36th run, the mineral solution was switched to the 0.12 M nitric acid containing 50 ng Y/ml and washing out of the interferents was observed in the next 12 consecutive runs. One run corresponded to 1.57 minute.

Calculations

Subtraction of the hydrogen peroxide mass spectral signal counts from each sample counts gave the blank-subtracted counts. Using the blank-subtracted signal counts, the four $^{67/64}Zn$, $^{67/66}Zn$, $^{67/68}Zn$, $^{67/70}Zn$ IR values were recalculated for each sample. The value obtained after subtraction of the baseline (zero time) IR from each IR value was divided by the natural Zn IR value to obtain the normalized IR (NIR) value. A data set of 4 normalized isotope ratios (67/64, 67/66, 67/68 and 67/70) x 163 time points x 2 treatments (extraction and nonextraction)obtained from 14 subjects after iv dose of ^{67}Zn was subjected to statistical analysis. All statistical analyses were carried out using the SYSTAT5 (version 5.2.1) Macintosh software (SYSTAT Inc., Evanston, IL).

From the semilogarithmic plot of NIRs versus plasma collection time (0 to 24 hours), the following kinetic parameters were calculated as follows: (a) the disappearance rate constant was calculated from the 30 min to 60 min slope, and (b) the zinc turnover rate by multiplying the initial slope with plasma zinc central compartment size obtained from the 'truncated model'. The 24-hour spot plasma pool size was calculated from the equation :

Spot pool size = Dose of iv tracer/NIR x Natural abundance of ^{67}Zn (Yokoi et al, 1997).

RESULTS AND DISCUSSION

Inductively coupled plasma-mass spectrometry has become a powerful alternative for the determination of isotope ratio measurements along with other well established techniques such as neutron activation analysis and thermal ionization and fast atom bombardment mass spectrometry. However, when biological material is analyzed by ICP-MS, potential interferences from polyatomic ions must be considered. These interfering polyatomic ions originate mainly from argon, nitrogen, and/or oxygen in combination with Na, S, Cl, and Ca, which are present at approximate concentration ranges of 3130-3370, 1120-1270, 2940-4120, and 92-109 mg/L in human serum, respectively (Vanhoe et al., 1989). Zinc has five isotopes: 64, 66, 67, 68, and 70. The most abundant isotope, ^{64}Zn

(48.9%), is interfered to a larger extent by polyatomic ions containing sulfur, oxygen, and calcium.

Polyatomic Interferences During the Isotope Ratio Measurements of the "Simulated Human Plasma" Mineral Solutions

In order to accurately calculate the actual polyatomic background signals generated during the ICP-MS analysis of the digested plasma solutions, the various "simulated human plasma" mineral solutions were subjected to the IR analyses of the routine ICP-MS instrumental conditions. Table 1 shows the equivalent concentrations of the interferents in the mineral solutions to naturally occuring Zn. Investigations of the mineral solutions were limited to the single mineral elements, and the mixture of two mineral elements and the mixture of all mineral elements. The investigation of the interaction among three mineral elements or more were omitted because of the statistical difficulties. The mixture of all minerals was tested because it was the closest to the digested human plasma. Ten times diluted solutions (in 0.12 M nitric acid) were chosen because we utilized similar dilutions in the on-going Zn nutritional study.

The ten times dilution of the digested human plasma contains approximately 100 ng Zn/ml. A careful study of the results from single element solutions in Table 1 indicates that the polyatomic interferences to ^{64}Zn by sulfur (3.61 ng/mL) and to ^{67}Zn by chlorine (2.58 ng/mL) alone are significant . On the other hand, the mixture of all mineral elements (S, Na, Cl, K, P and Ca) which is approximately equivalent to the digested human plasma, largely interfered only with ^{64}Zn (6.66 ng/mL) and ^{70}Zn (8.51 ng/mL). However, the interferences to ^{66}Zn, ^{67}Zn, and ^{68}Zn are minimal containing 0.90, 0.94, and 0.39 ng/mL, respectively.

It is obvious that interactions among mineral elements evoked the shift of the interferents from 67 to 70 of the atomic mass units. The mixture of Na - Cl, S - Cl, Cl - K and Na - P evoked the interference with ^{70}Zn. The mixture of S - Cl, Na - Cl reduced the interference with ^{67}Zn. These results suggest that co-presence of Na or S affects the chemical reaction of Cl in argon plasma, and the major interferent is shifted from $^{35}Cl^{16}O_2$ (atomic mass 67 coming from Cl solution) to $^{35}Cl_2$. Table 2 summarizes the possible polyatomic species generated by the introduction of single and various combinations of mineral solutions to the inductively coupled argon plasma. Figure 1 shows the changes in Zn-equivalent concentrations for the interferents to Zn isotopes found in the continuous

ICP-MS acquisitions of the ten times diluted "simulated plasma" mineral mixture containing 120 ppm S, 360 ppm Na, 330 ppm Cl, 18.9 ppm K, 14.1 ppm and 9.9 ppm Ca dissolved in 0.12 N nitric acid.

Comparison of Isotope Ratio Results from Human Plasma Samples - "Extraction" versus "Nonextraction"

Table 3 lists the range of normalized isotope ratios (NIRs) for the four isotope ratios chosen. When Zn in sample is extracted, Zn isotopes 64, 66 and 68 can be used as a denominator isotope to calculate normalized isotope ratio. However, the low abundance and counts of ^{70}Zn does not allow an accurate measurement of normalized isotope ratio even after the extraction of Zn. As expected, all the NIR values were found to be the lowest after 9 days of intravenous administration of ^{67}Zn and highest at 5 minutes after injection. Negative values are irrational because all NIRs were obtained only after the administration of ^{67}Zn. The frequency of negative values for NIR was found to be very low; 2 out of 163 values (1.2%) for NIR-A $^{67/70}Zn$ and 7 out of 163 values (4.3%) for NIR-B $^{67/70}Zn$. Negative values were not observed for NIRs obtained from $^{67/66}Zn$ and $^{67/68}Zn$.

Figure 2 shows correlation plots of normalized isotope ratios of $^{67/68}Zn$ versus $^{67/66}Zn$ for extracted (A, $r^2 = 0.998$) and nonextracted (B, $r^2 = 0.992$) plasma samples. Only at very low NIR values, the data points tend to deviate from linearity for the nonextracted samples. The value of $r^2 = 0.992$ obtained for nonextracted samples is very close to $r^2 = 0.998$ for extracted samples and acceptable for kinetics. Table 4 summarizes the correlations (r^2) between different NIRs obtained from extracted samples only using simple linear regression and double logarithmic (power function fitting) plots. As expected, the correlations are high for all the four isotopes, 64, 66, 67 and 68, due to the removal of the interfering polyatomic background ions during the extraction of Zn. Figure 3. shows the correlations of normalized isotope ratios for $^{67}Zn/^{68}Zn$ (A, $r^2 = 0.987$) and $^{67}Zn/^{66}Zn$ (B, $r^2 = 0.976$) for extracted versus nonextracted plasma samples.

Table 5 compares the normalized isotope ratios obtained from both the extracted (NIR_A) and nonextracted (NIR_B) samples using simple linear regression and double logarithmic (power function fitting) plots. NIR_B calculated from 67/68 and 67/66 agrees very well with NIR_A. The extent of agreement of A and B batches for 67/68 is followed by 67/66. As expected from the results of the detailed investigation of polyatomics

interferences for ^{64}Zn and ^{70}Zn (Tables 1 and 2, and Figure 1), NIR_B calculated from 67/64 and 67/70 poorly agreed with NIR_A.

In summary, the regression analyses values (r^2, the slope "a", and the intercept "b") for NIR correlations from both the "extraction" and "nonextraction" methods show high correlations for $^{67/68}Zn$ and $^{67/66}Zn$. Such high correlations for "nonextracted" samples can be routinely achieved by: (a) keeping the resolution of the mass spectrometer between 0.8 and 0.9 amu, (b) cleaning the skimmer/sampling cones, torch, and the nebulizer prior to analysis of each batch of samples, and (c) passing nitric acid (1%) between the samples until the ^{89}Y (internal standard) signal reaches below 200 counts . The resolution of the mass spectrometer is crucial to reduce the unexpected backgrounds.

However, the correlations are poor for $^{67/64}Zn$ (r^2 = 0.838) and $^{67/70}Zn$ (r^2 = 0.747) (see Table 5) due to sulfur and oxygen polyatomic (mostly $^{32}S^{16}O_2$ and $^{32}S_2$) backgrounds at ^{64}Zn mass and shifting of the major interferent, $^{35}Cl^{16}O_2$ (atomic mass 67 coming from Cl solution), to $^{35}Cl_2$.(atomic mass 70) in combination with very low natural abundance for ^{70}Zn, respectively. Considering the possibility of isobaric interferences generated during the ionization processes of the digested plasma samples inside the inductively coupled plasma of the ICP-MS coupled with this detailed investigation indicate that $^{67/68}Zn$ and $^{67/66}Zn$ NIRs with least possibility of polyatomic backgrounds obtained from "nonextracted" samples are sufficient for routine Zn kinetic analysis using ^{67}Zn enriched isotope. It should be pointed out that since the polyatomic backgrounds at atomic mass 67 are shifted to atomic mass 70, the "nonextraction" procedure may not be suitable for Zn kinetic analysis using ^{70}Zn enriched isotope.

Ideally extraction of zinc as a purification step appears desirable. The Zn extraction procedure, however, involves many steps and some steps are susceptible to contamination. Zinc is ubiquitous in the environment and contamination of Zn from the environment is inevitable during extraction. Our laboratory experience from analysis of several hundred samples for isotope ratio measurements emphasizes the benefit of analysis of both an "extracted" and "nonextracted" specimens. From such rigid analyses the evidence of lack of contamination can be easily verified.

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Table 1. The equivalent concentrations (ng/ml) of the interferents in the mineral solutions to naturally occuring zinc.

	Mass number				
	64	66	67	68	70
Single mineral					
S	3.61	0.38	0.01	0.07	-1.45
Na	0.59	0.48	0.28	0.49	0.60
Cl	0.25	0.25	2.58	0.30	1.59
K	-0.08	-0.09	-0.15	-0.10	-0.47
P	-0.09	-0.11	-0.14	-0.11	-0.63
Ca	-0.08	-0.09	-0.15	-0.09	-0.58
Mixture of two minerals					
S - Na	5.53	0.55	0.22	0.10	0.00
S - Cl	5.08	0.65	0.94	0.29	6.82
S - K	4.19	0.17	-0.13	-0.19	-0.62
S - P	3.96	0.32	-0.02	-0.03	-0.58
S - Ca	3.45	0.13	-0.05	-0.15	-0.80
Na - Cl	1.02	0.63	1.00	0.65	13.35
Na - K	0.50	0.27	0.40	0.29	1.27
Na - P	0.74	0.38	0.67	0.40	2.15
Na - Ca	0.45	0.31	0.48	0.33	0.83
Cl - K	0.17	0.16	2.78	0.21	3.10
Cl - P	0.22	0.22	2.65	0.25	1.34
Cl - Ca	0.15	0.15	1.94	0.17	1.14
K - P	-0.08	-0.09	0.08	-0.08	-0.23
K - Ca	0.00	-0.01	0.11	0.00	-0.23
P - Ca	0.07	0.06	0.32	0.08	-0.23
Mixture of all minerals	6.66	0.90	0.94	0.39	8.51
Sum of the single minerals	4.19	0.82	2.43	0.57	-0.95

Each solution contains one tenth of the mineral concentration(s) found in the representative human plasma.

S: 120 μg/ml S as H_2SO_4. Na: 330 μg/ml Na as $NaNO_3$. Cl: 360 μg/ml as HCl. K: 18.9 μg/ml K as KNO_3. P: 14.1 μg/ml as $(NH_4)_2HPO_4$. Ca: 9.9 μg/ml Ca as $Ca(NO_3)_2$.

All mineral solutions were prepared in 0.12 M HNO_3.

Table 2. Possible polyatomics generated by the introducion of the mineral solutions to inductively coupled argon plasma.

	Mass number				
	64	66	67	68	70
Single mineral					
S	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
Na	-	-	-	-	-
Cl	-	-	$^{35}Cl^{16}O_2$	-	-
K	-	-	-	-	-
P	-	-	-	-	-
Ca	-	-	-	-	-
Mixture of two minerals					
S - Na	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
S - Cl	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	$^{35}Cl_2$
S - K	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
S - P	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
S - Ca	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	-
Na - Cl	-	-	-	-	$^{35}Cl_2$
Na - K	-	-	-	-	-
Na - P	-	-	-	-	$^{23}Na^{31}P^{16}O$
Na - Ca	-	-	-	-	-
Cl - K	-	-	$^{35}Cl^{16}O_2$	-	$^{35}Cl_2$
Cl - P	-	-	$^{35}Cl^{16}O_2$	-	-
Cl - Ca	-	-	-	-	-
K - P	-	-	-	-	-
K - Ca	-	-	-	-	-
P - Ca	-	-	-	-	-
Mixture of all minerals	$^{32}S^{16}O_2$, $^{32}S_2$	-	-	-	$^{35}Cl_2$
Sum of the single minerals	$^{32}S^{16}O_2$, $^{32}S_2$	-	$^{35}Cl^{16}O_2$	-	-

Each solution contains one tenth of the mineral concentration(s) found in the representative human plasma.
S: 120 µg/ml S as H_2SO_4. Na: 330 µg/ml Na as $NaNO_3$. Cl: 360 µg/ml as HCl. K: 18.9 µg/ml K as KNO_3. P: 14.1 µg/ml as $(NH_4)_2HPO_4$. Ca: 9.9 µg/ml Ca as $Ca(NO_3)_2$.

Table 3. The range of Normalized Isotope Ratios (NIRs)

	"Extracted" Samples			
	67/64	**67/66**	**67/68**	**67/70**
Minimum	0.04	0.06	0.06	-0.43
Median	0.78	0.75	0.72	0.68
Maximum	14.33	13.67	12.91	12.89

	"Nonextracted" Samples			
	67/64	**67/66**	**67/68**	**67/70**
Minimum	-0.160	0.05	0.06	0.05
Median	0.53	0.71	0.75	0.69
Maximum	12.42	13.23	12.69	12.15

Minimum NIR was found 9 days after i.v. dose of ^{67}Zn.
Maximum NIR was found 5 minutes after i.v. ^{67}Zn administration.
Negative values are irrational because all NIRs were obtained after administration of ^{67}Zn.

Table 4. Correlations (r^2) between different NIRs obtained from extracted samples using simple linear regression and double logarithmic (power function fitting) plots

	Simple Linear Regression Plot		
	67/66	**67/68**	**67/70**
67/64	0.996	0.994	0.887
67/66		0.999	0.889
67/68	-		0.802
	Double Logarithmic Plot		
	67/66	**67/68**	**67/70***
67/64	0.996	0.991	0.786
67/66		0.998	0.794
67/68			0.893

*Negative values were removed for calculation.

Table 5. Comparison of normalized Zn isotope ratios (NIRs) obtained from extracted (A batch, NIR_A) and nonextracted (B batch, NIR_B) samples using simple linear regression and power function fitting (double logarithmic) plots

	Simple Linear Plot		
NIR	**r2**	**a**	**b**
67/64	0.838	0.059	0.175
67/66	0.983	0.985	0.040
67/68	0.985	0.964	0.035
67/70	0.747	0.907	0.132
	Double Logarithmic Plot		
NIR	**r2**	**a**	**b**
67/64*	0.838	1.237	0.773
67/66	0.976	1.023	0.958
67/68	0.985	0.987	1.001
67/70*	0.747	0.966	0.903

Regression equation for the simple linear equation is: NIR_A = a NIR_B + b, where 'a' and 'b' are the slope and the intercept, respectively. For perfect correlations, 'a' should be equal to 1 and and 'b' should be zero.

Regression equation for the double logarithmic plot is: NIR_A = a NIR_B power b. If both the values completely agree, then 'a' and 'b' should each be equal to1.

*Negative values are removed because they do not allow fitting.

Legends for Figures

Figure 1. Changes in Zn-equivalent concentrations for the interferents to Zn isotopes found in the continuous ICP-MS acquisition of the ten times diluted "simulated plasma" mineral mixture containing 120 ppm S, 360 ppm Na, 330 ppm Cl, 18.9 ppm K, 14.1 ppm and 9.9 ppm Ca dissolved in 0.12 N nitric acid.

Figure 2. Correlation plots of normalized isotope ratios of $^{67}Zn/^{66}Zn$ versus $^{67}Zn/^{68}Zn$ for extracted (A) and nonextracted (B) plasma samples.

Figure 3. Correlation plots of normalized isotope ratios for $^{67}Zn/^{68}Zn$ (A) and $^{67}Zn/^{66}Zn$ (B) for extracted versus nonextracted plasma samples.

Figure 1

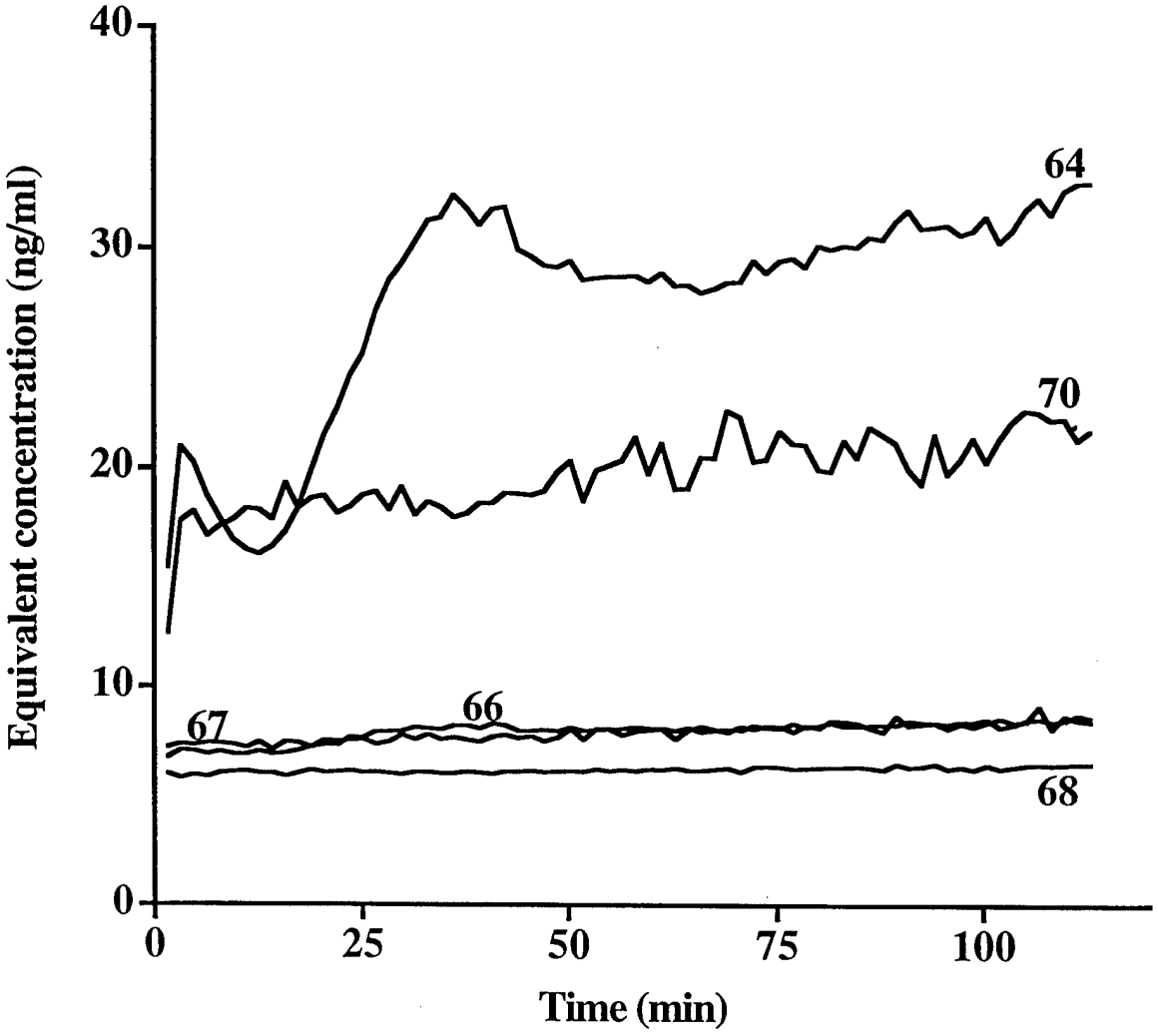

40
30
20
10
0
Equivalent concentration (ng/ml)
64
70
66
67
68
0
25
50
75
100
Time (min)

Figure 2

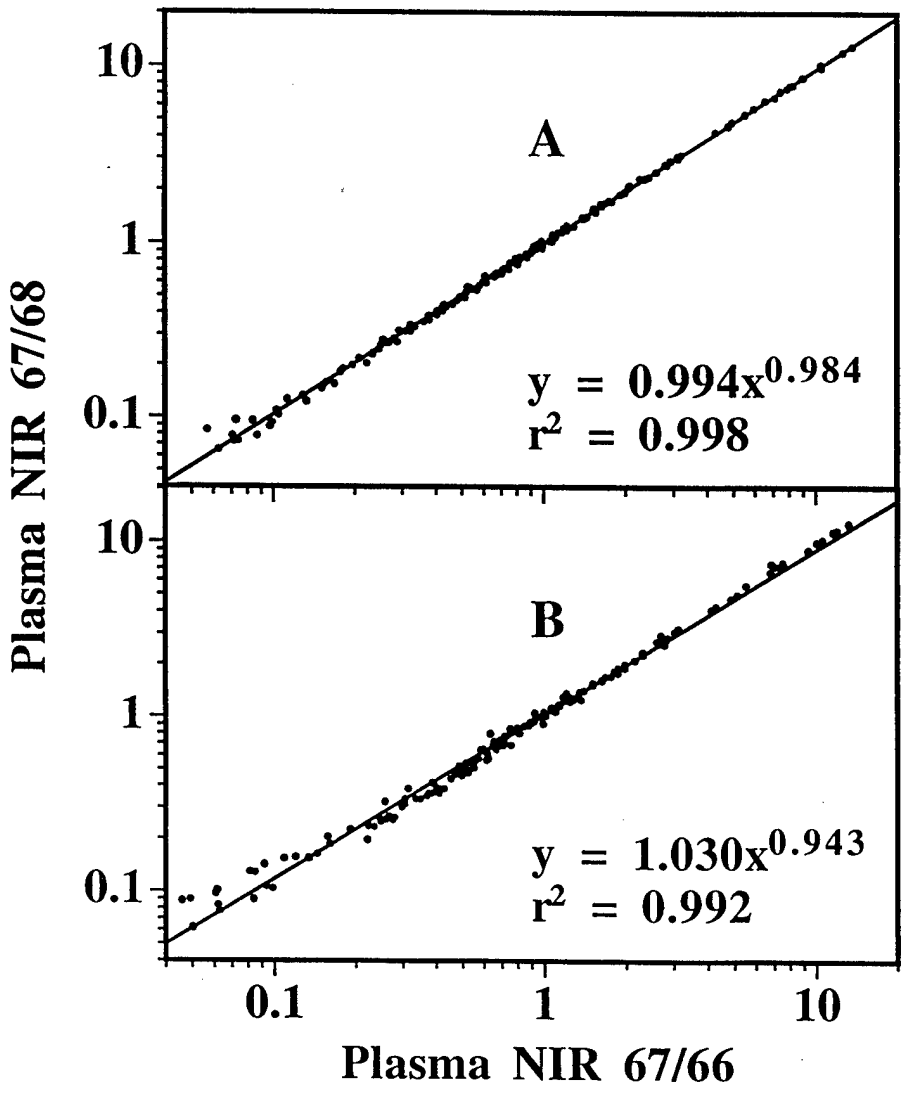
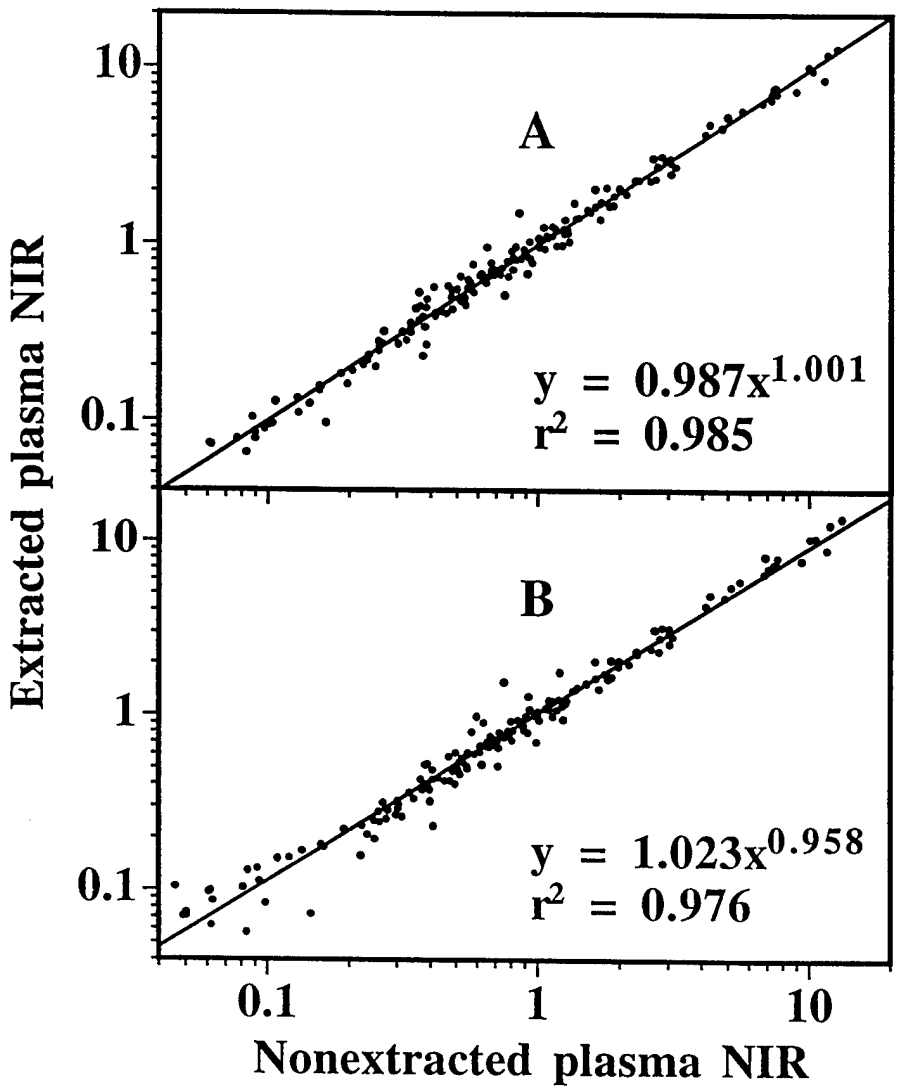

10
1
0.1
A
y = 0.994x^0.984
r^2 = 0.998
10
1
0.1
B
y = 1.030x^0.943
r^2 = 0.992
0.1
1
10
Plasma NIR 67/68
Plasma NIR 67/66

Figure 3


A
y = 0.987x^1.001
r^2 = 0.985
B
y = 1.023x^0.958
r^2 = 0.976
10
1
0.1
Extracted plasma NIR
Nonextracted plasma NIR

THE EXCHANGEABLE ZINC POOL SIZE RELATES TO LEAN BODY MASS AND SKELETAL MUSCLE MASS IN HUMANS

ABSTRACT

The rapidly exchangeable zinc (Zn) pool size (EZP) is believed to represent Zn that is available to tissues for current physiological needs. However, its relationship to body composition is unknown. To ascertain these relationships we measured EZP (estimated from the 24-h "truncated" exponential kinetic model and the 24-h spot plasma Zn pool) and Zn turnover rate (TR; estimated from the product of plasma Zn and the initial slope of the disappearance curve after i.v. administration of ^{67}Zn). Twenty seven healthy volunteers (5 males and 22 females) were studied. Ages ranged from 22-63 years, body mass index from 19-35.3 kg/m^2, and EZP from 102-251 mg. Zn isotope ratios in plasma were determined by inductively coupled plasma-mass spectrometry. Lean Body Mass (LBM) was derived from total body water which was measured by bioelectrical impedance. Skeletal Muscle Mass (SMM) was estimated from the 24-h urinary creatinine excretion (Wang et al, AJCN 1996;63:863). EZP obtained from the 24-h kinetic model and the 24-h spot plasma Zn pool were identical ($r= 0.994$, $p<0.0001$). Highly significant correlations were found between EZP and SMM ($r=0.900$, $p<0.0001$) and LBM ($r=0.81$, $p<0.0001$). TR correlated with EZP ($r=0.83$, $p<0.0001$), with LBM ($r=0.87$, $p<0.0001$), and with SMM ($r=0.82$, $p<0.0001$). Lesser correlations were found between EZP and fat mass ($r=0.646$, $p=0.01$). These results suggest that skeletal muscle is an important contributor to EZP.

INTRODUCTION

Wastney *et al* (1986) analyzed human Zn kinetics using ^{65}Zn and developed a complex multicompartment system. The concept of "the exchangeable Zn pool" (EZP) within 48 hours after tracer administration was proposed. This readily exchangeable pool accounts for only the minority (<10%) of total body zinc, but is assumed to be responsible for its physiological functions. Miller et al (1994) estimated EZP from the extrapolation of data in the semilogarithmic plot of isotopic enrichment vs time obtained between 3 - 9 days after administration of stable Zn isotopes. They found a correlation between EZP and body weight and dietary zinc intake. We developed a method for evaluation of EZP based on a much shorter (24-h) observation period after ^{67}Zn intravenously, and compared the results with indicators of body composition.

METHODS

1. Inject 2 mg ^{67}Zn intravenously to 27 human subjects (5 men and 22 women) and collect blood samples at baseline (before administration) and 5, 15, 30, 40, 50, 60, 90 minutes, 2, 6, 12, and 24 hours later.

2. Digest the specimens and measure the Zn isotope ratio ^{67}Zn/^{68}Zn by ICP-MS (Plasma Quad, VG Instruments) based on Yokoi et al (1994a; 1994b).

3. Subtract baseline from the measured isotope ratio and divide the ratio by natural Zn isotope ratio to obtain the normalized isotope ratio (NIR).

4. Calculate approximation of EZP from 5 min - 24 h plasma data.

5. Calculate plasma Zn turnover rate by multiplying the initial slope of the semilogarithmic plot of the normalized isotope ratio vs time and plasma Zn (central) compartment size.

6. Measure (in the morning after 8 hrs fast) body composition, i.e., body weight, lean body weight (bioelectrical impedance), 24-h urinary creatinine excretion (Jaffé method), and compare these with EZP and plasma Zn TR.

OBJECTIVES

To evaluate the relationship in normal humans between **Rapidly Exchangeable Zn Pool** (EZP) and:

- plasma Zn turnover rate (TR)
- body composition.

RESULTS AND DISCUSSION

The anthropometric and kinetic parameters of the subjects are shown in **Table 1**. All but two female subjects had normal plasma Zn concentration (≥ 700 ng/ml).

Figure 1 shows the correlation between 24-h spot plasma Zn pool and the rapidly exchangeable Zn pool (EZP) calculated from the 24-h truncated exponential kinetic model based on the 5 min to 24 h data. The 24-h spot plasma Zn pool appears to be a practical and good estimate of EZP, although calculated from just a single plasma sample, obtained 24 hours after i.v. administration of ^{67}Zn.

Body weight of the subjects correlated well with EZP **(Figure 2)**. A highly significant correlation with negligible intercept indicates that the normal human body has a quite constant amount of EZP per body weight.

Lean body weight of the subjects correlated well with EZP **(Figure 3)**.

The 24-h urinary creatinine excretion was highly correlated with EZP ($r=0.9$, $p<0.0001$). The skeletal muscle mass was calculated from this 24-h urinary creatinine excretion using the revised Forbes equation (Wang et al. 1996). As a result, the intercept of the regression line became negligible, suggesting that a considerable part of EZP may exist within the skeletal muscle mass **(Figure 4)**.

Correlation between fat mass and EZP ($r=0.6$, $p=0.013$) was poor compared to lean body weight and estimated skeletal muscle mass, suggesting less contribution of fat mass to EZP.

Figure 5 shows the correlation between EZP and plasma Zn turnover rate. It is interesting that the plasma Zn turnover rate is determined by the initial slope and the extrapolated intercept while EZP is actually determined by the data of 24-h spot plasma. Because the plasma Zn turnover rate is about 300 to 600 mg/day and much higher than the excretion rate of Zn (<10 mg/day), it is closely identical to the Zn uptake rate by tissues. EZP reflects metabolically active tissue mass as shown above. It appears that we observed the same metabolically active mass both initially (TR) and 24 hours later (EZP) through behaviour of the tracer.

As expected from the highly significant correlation between EZP and plasma Zn TR, plasma Zn TR highly correlated with body weight ($r=0.77$, $p<0.0001$), lean body weight **(Figure 6)** and estimated muscle mass **(Figure 7)**.

Table 1.
Anthropometric and kinetic parameters of the subjects (Mean±SD).

	Females (n=22)	Males (n=5)
Age (years)	30±4.6	41±14.7
Body weight (kg)	63±11.6	80.5±8.5
Body height (m)	1.65±0.07	1.76±0.06
Body mass index (kg/m^2)	23.2±4.5	26.1±3.1
Lean body weight (kg)	45.6±5.8	62±4.3
Plasma zinc (ng/ml)	783±82	927±89
Turnover rate (mg/day)	357±67	549±54
Exchangeable Zn Pool (mg)	151.8±33	221±25

Figure 1: Relation between 24 hr. plasma Zn Pool and Exchangeable zinc Pool (EZP)

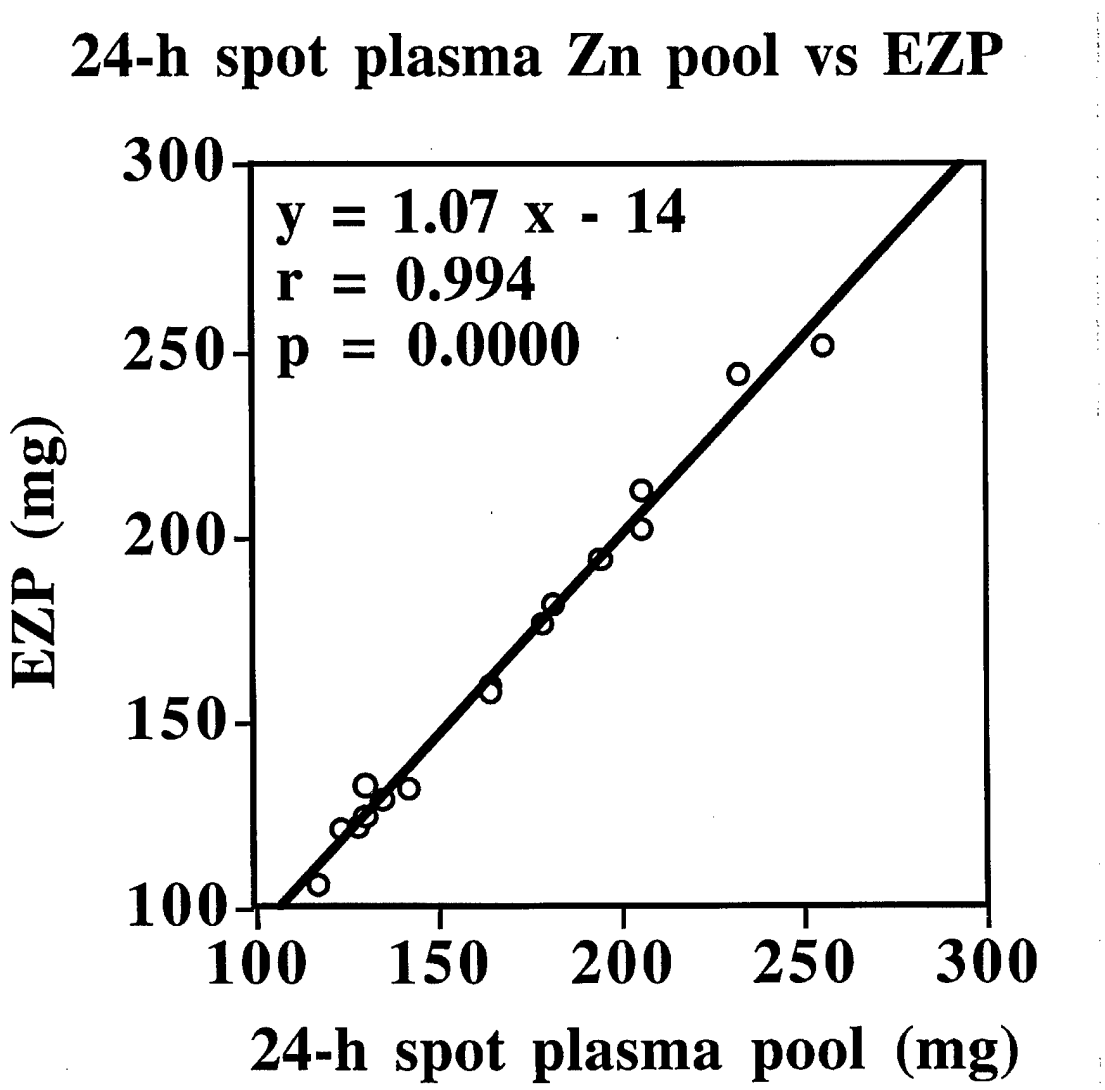


Figure 2: Relation between Body Weight and Exchangeable zinc Pool (EZP)

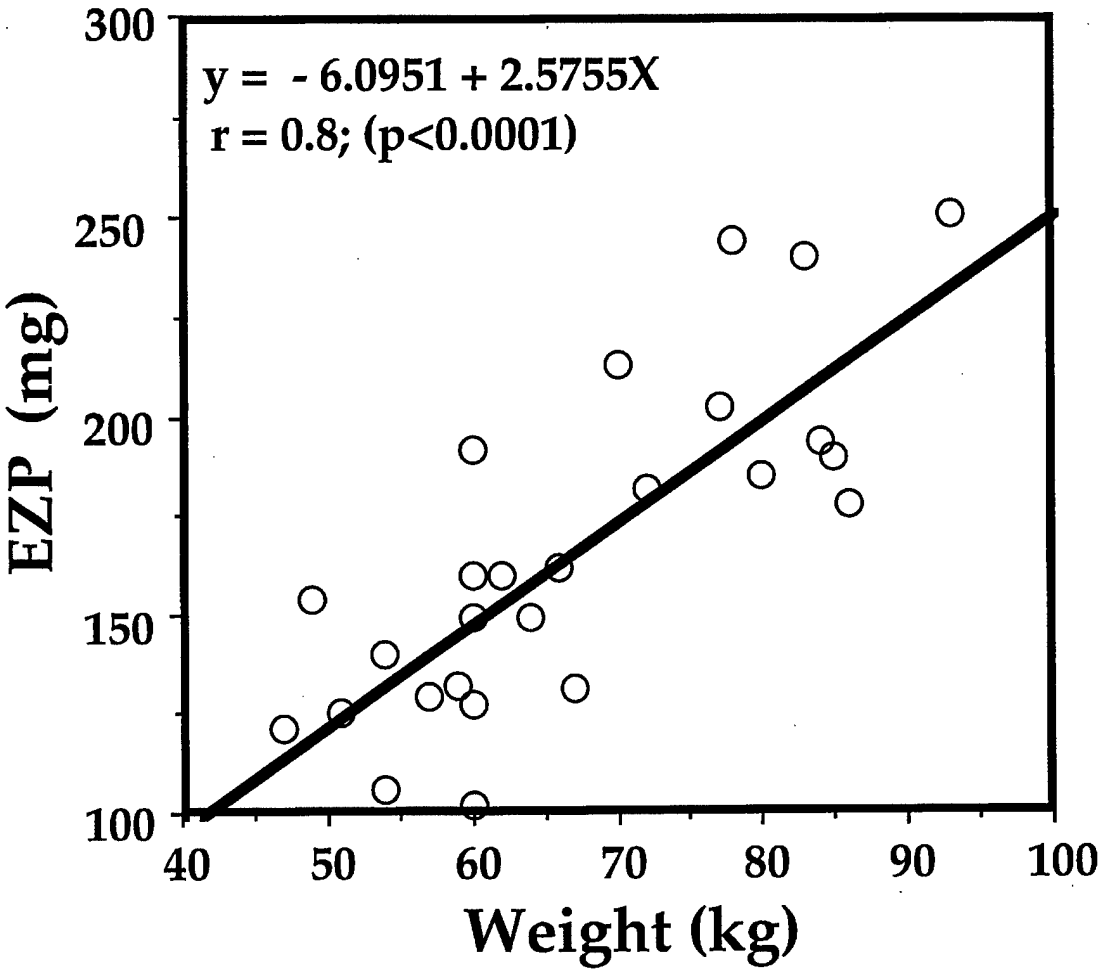


Figure 3: Relation between Lean Body Weight and Exchangeable zinc Pool (EZP)

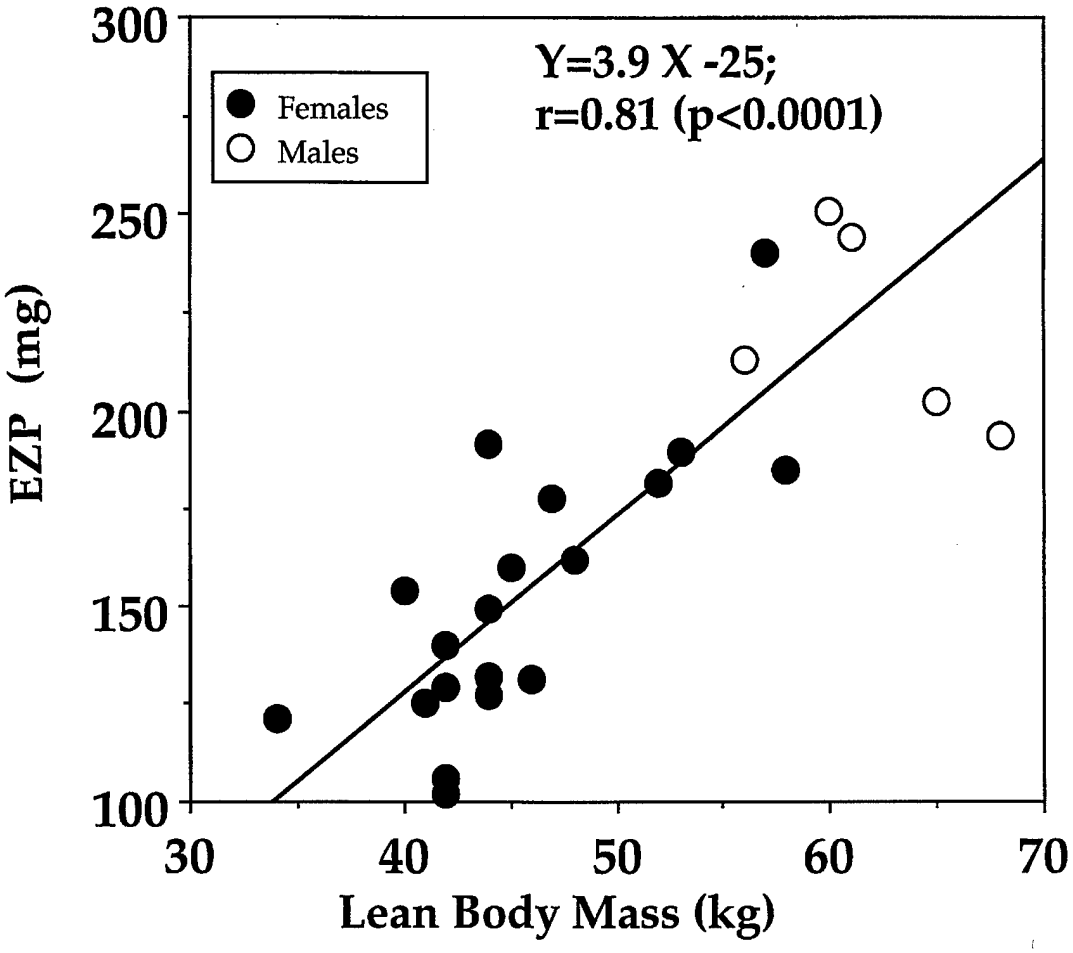


Figure 4: Relation between estimated skeletal muscle mass and Exchangeable zinc Pool (EZP)

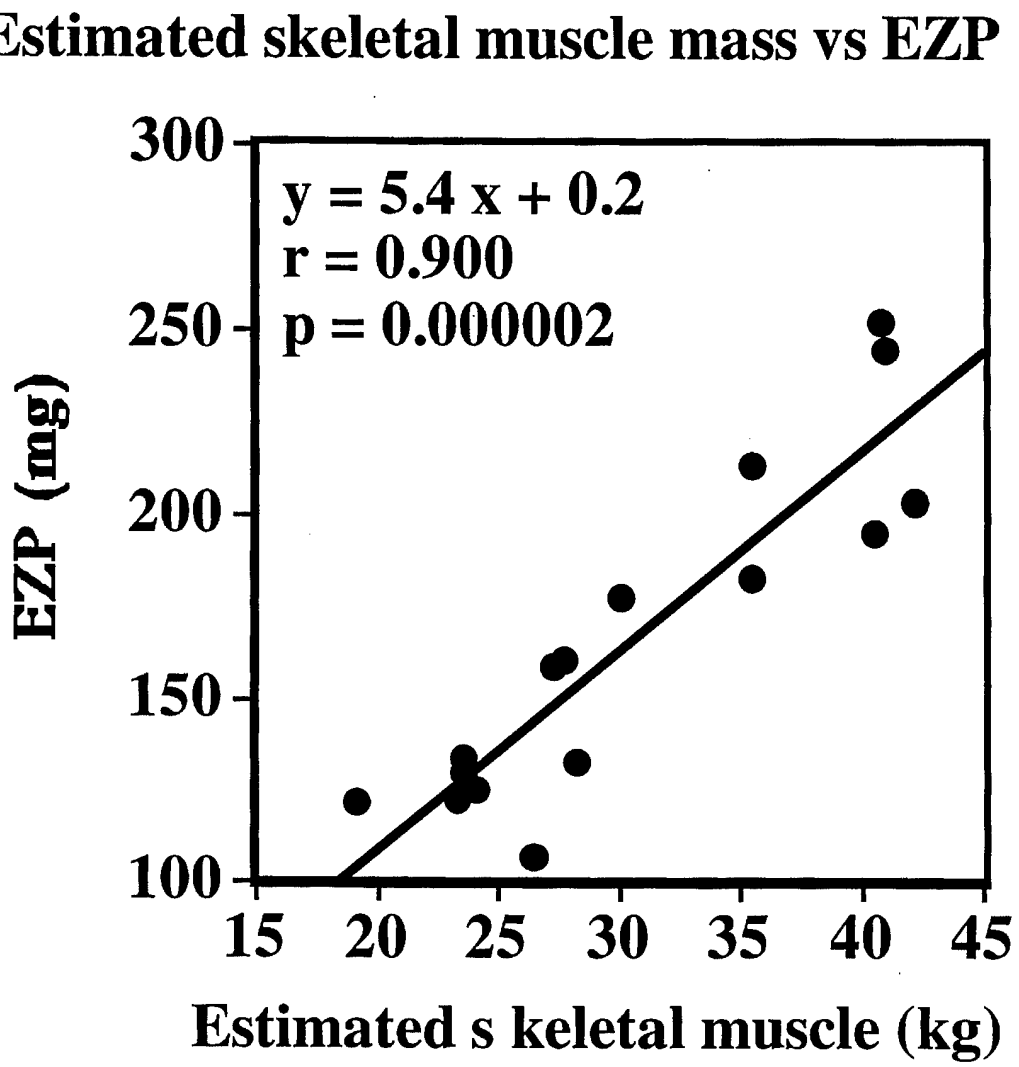


Figure 5: Relation between plasma zinc turnover rate (Zn TR) and Exchangeable zinc Pool (EZP)

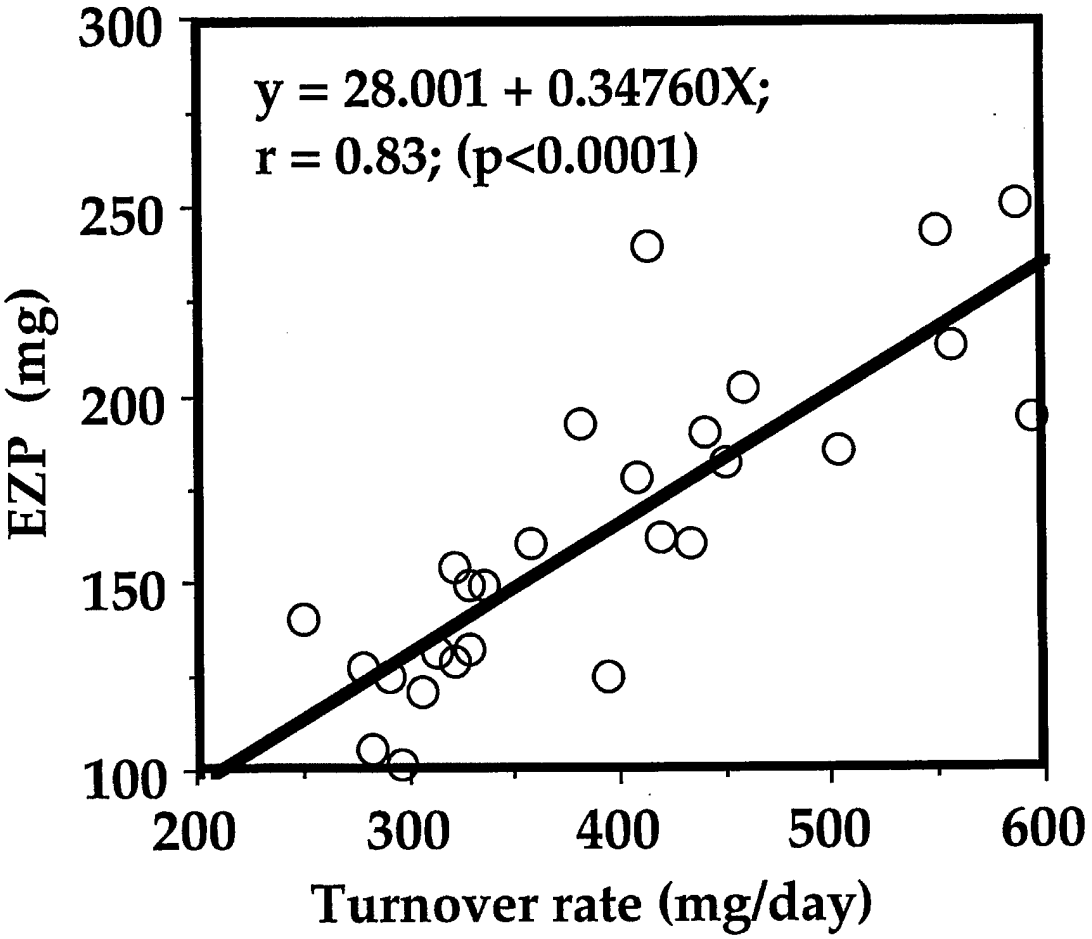


Figure 6: Relation between Lean Body Weight and Plasma zinc turnover rate (Zn TR)

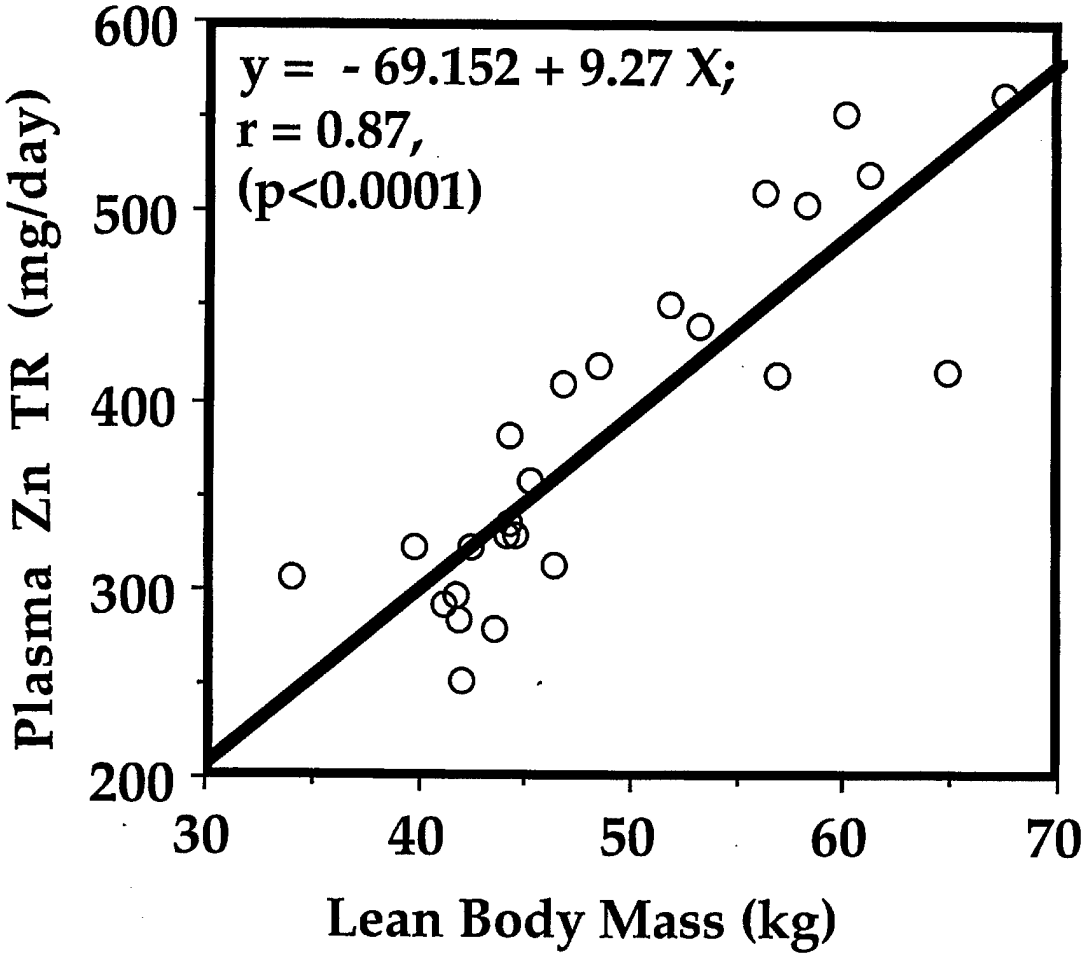
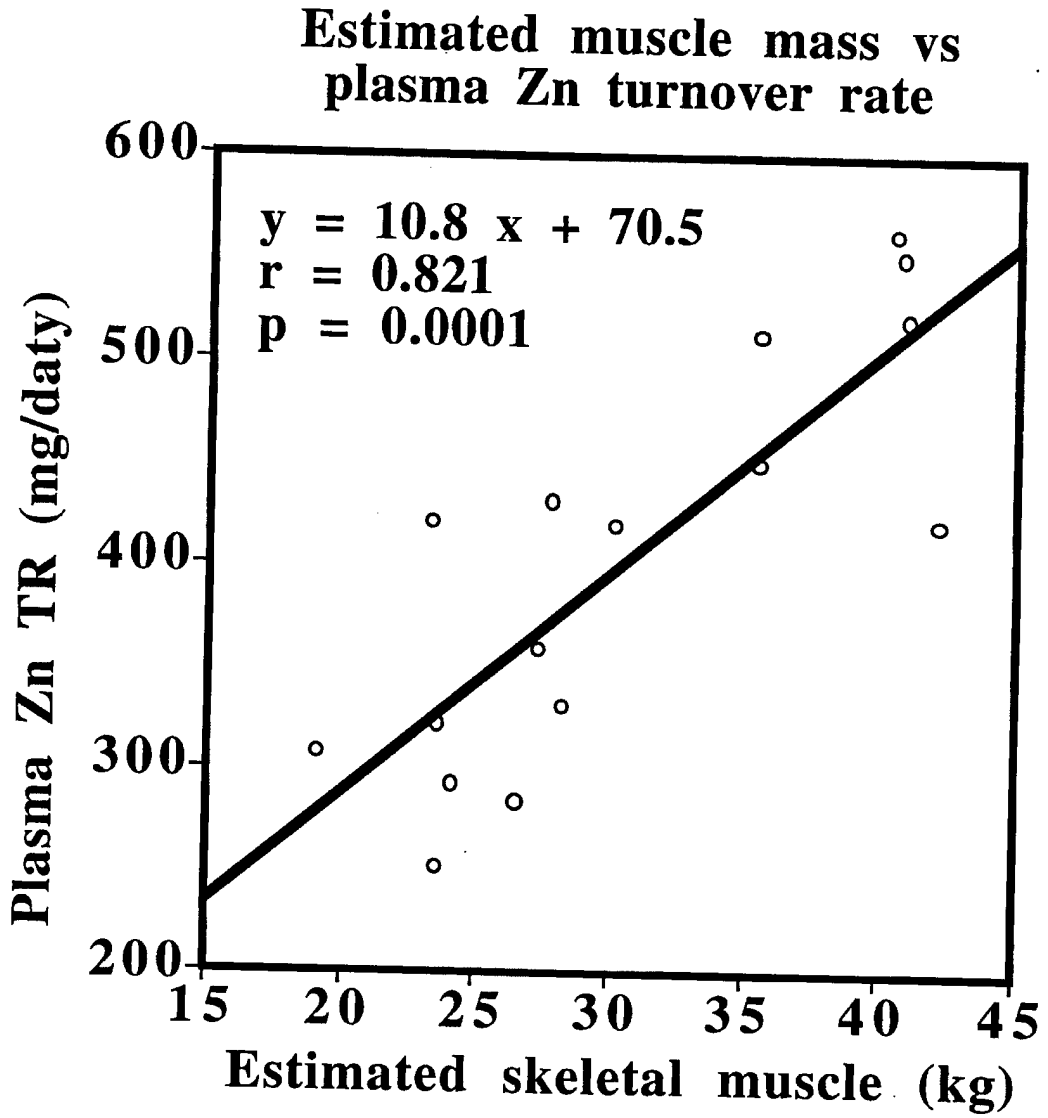


Figure 7: Relation between Plasma zinc turnover rate (Zn TR) and estimated skeletal muscle mass



CONCLUSION

1. 24-h spot plasma Zn pool is a good approximation of EZP.

2. EZP correlates well with body weight, lean body weight and skeletal muscle mass (derived from 24-h urinary creatinine excretion) rather than fat mass, suggesting that skeletal muscle and visceral mass are the major contributor to EZP.

3. Highly significant correlation between EZP (static index) and plasma Zn TR (dynamic index) indicates that both indices originate from the same metabollicaly active mass.

Literature

Miller LV, Hambidge KM, Naake VL, Hong Z, Westcott JL, Fennessey PV (1994) Size of the zinc pools that exchange rapidly with plasma zinc in humans: Alternative techniques for measuring and relation to dietary zinc intake. Journal of Nutrition **124**:268-276.

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Yokoi K, Alcock NW, Sandstead HH (1994b) Iron and zinc nutriture of premenopausal women: Associations of diet with serum ferritin and plasma zinc disappearance and of serum ferritin with plasma zinc and plasma zinc disappearance. Journal of Laboratory and Clinical Medicine **124**:852-861.

Wang Z-M, Gallagher D, Nelson ME, Matthews DE, Heynsfield SB (1996) Total-body skeletal muscle mass: evaluation of 24-h urinary creatinine excretion by computerized. American Journal of Clinical Nutrition **63**:863-9.

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Acknowledgment

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Principal Investigator: Sandstead, Harold H.
Contract No. DAMD17-95-C-5112

QUARTERLY REPORT

1. Contract No.: DAMD17-95-C-5112 2. Report Date: 1-30-97
3. Reporting Period from: 9-22-96 to 12-22-96
4. PI: Harold H. Sandstead 5. Telephone No. (409) 772-4661
6. Institution: The University of Texas Medical Branch
7. Project Title: Repletion of Zinc and Iron deficiencies improves cognition of premenopausal women.
8. Current Staff, with percent effort of each on project:

Harold H. Sandstead	15 %	Nancy W. Alcock	10 %
VM Sadagopa Ramanujam	25 %	Hari H. Dayal	10 %
Norman G. Egger	100 %	Jackie Callies	60 %
Katsuhiko Yokoi	25 %		

10. Administrative and logistical matters.

    a. From the beginning of the project 185 potential subjects have contacted us by phone to learn about the project. Sixty eight of these respondents expressed interest and met criteria for undergoing screening. They were seen by a physician, informed in detail, completed the medical and dietary questionnaires, were examined, and had their blood chemistries determined. Of these women 22 met the criteria for inclusion and have completed the assessment of zinc status through measurement of kinetics and white blood cell and platelet zinc. Twelve subjects completed the baseline neuropsychological evaluation. Three of them are now receiving the first treatment. Four subjects have finished the first treatment and first follow-up evaluation, and are now receiving the second treatment. One subject has completed both treatments and all follow-up evaluations. Two subjects dropped out after the first treatment (they indicated they didn't have time to participate).

    b. Results of the neuropsychological measurements have been transferred to our collaborator at the USDA ARS Grand Forks Human Nutrition Research Center for evaluation. Blood and urine samples for various assays have been sent to collaborators.

    c. Recruitment of iron and zinc deficient subjects is seriously behind our goal. Only two of the 68 individuals screened have displayed low serum ferritin concentration. We are perplexed by this. In our 1992 study 34 individuals with low serum ferritin were easily identified, and in fact we had difficulty finding subjects with normal serum ferritin concentrations. Data from NHANES-II indicate at least 25 % of young US women have serum ferritin concentrations $\leq$ 14 ng/mL.

    d. To increase our chances of finding women with low iron and zinc status we are now targeting ads at vegetarians.

    e. We shared our concerns about recruitment with the contracting officer and proposed changes that may improve recruitment. With her approval we have modified the protocol and have submitted the modifications to our IRB for approval. The changes are: 1. shortening the treatment intervals to 4 weeks each: 2. including obese and low income persons in the study; 3. offering three options for participation, a. the entire study including zinc kinetics and measurements of effects of treatments on neuropsychological functions, b. measurements of kinetics only, c. measurement of responses to treatment only.

    f. We suggested to the Contracting Officer that given the difficulty of finding subjects, 18 months may be too short a time to complete evaluation of 60 low iron subjects. We suggested an extension might be needed and that consideration might appropriately be given to finding support for staff that are carrying out the day-to day aspects of the study.

    g. The research group continued to meet weekly to review progress.

11. Scientific progress for the quarter in terms of the tasks or objectives listed in the statement of work for this contract. Explain deviations where this isn't possible. Include data where possible.

    a. During this quarter zinc kinetics has been measured on 12 additional subjects. Chemical analysis of the plasma and urine samples is complete for 10 of them. Thus

during the past 15 months zinc kinetics have been measured in 22 subjects and 6 controls (male staff).

b. Based on the above data 3 abstracts were submitted to the April 1997 Experimental Biology, American Society for Nutrition Sciences meeting in New Orleans, Louisiana. These abstracts describe improvements in the ICP-MS method, the derivation of the rapidly exchangeable zinc pool and the relation of the exchangeable zinc pool to the plasma zinc disappearance rate from 30-90 minutes after injection, the lean body mass, and the urinary creatinine excretion (Addendum-I).

c. Analysis of white blood cell zinc: This quarter white blood cells and platelets were isolated from 6 subjects. The yield for the fractions was:

Lymphocytes (3.33 - 7.79) x 10^3/ul suspension
Granulocytes (1.31 - 8.72) x 10^3/ul suspension
Platelets (365 - 1134) x 10^3/ul of suspension

The percent yield of the individual cells in the suspension counted was 73.2 - 91.6 for lymphocytes, and 81.6-96.3 for granulocytes. Contamination of these fractions with platelets did not exceed 3 x 10^3/ul, and therefore the contribution of zinc from platelets was minimal. An aliquot of each suspension was digested with hydrogen peroxide, redissolved in 0.5N-HNO_3, and stored at -20^0 C. Analysis for zinc by graphite furnace atomic absorption is currently in progress.

d. Analysis of plasma beta-hydroxybuterate: This analyte has been measured in all subjects studied to date, and all values are in the normal range. No difference in the value was obtained from specimens stored at -20^0 C or -75^0 C for up to 119 days.

e. The PI was invited to present this project at a conference concerning Preparedness of Military Women of the Food and Nutrition Board Committee on Military Medicine. The extended abstract is attached in Addendum-II

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

Select and randomize at least 5 low ferritin subjects according for the treatment trial.

ADDENDUM I

Abstracts submitted to the April 1997 Experimental Biology, American Society for Nutrition Sciences meeting in New Orleans, Louisiana.

MATHEMATICAL MODELS FOR TWENTY-FOUR-HOUR AND NINE-DAY ZINC KINETICS IN HUMANS. K. Yokoi, V. M. Sadagopa Ramanujam, N. G. Egger, H. H. Dayal, N. W. Alcock and H. H. Sandstead. Univ. of Texas Med. Br., Galveston, TX 77555-1109. Kyoto Univ. Grad. Sch. of Med., Kyoto 606-01, Japan.

Twenty-four-hour zinc (Zn) kinetics concordant with the nine-day kinetics was developed to show the validity of the 24-h spot plasma Zn pool as a practical indicator of the so-called rapidly exchangeable Zn pool size (EZP). We compared kinetic parameters after iv dose of ^{67}Zn derived from 0 - 9 days (9-d model) and from 0 - 24 hours (24-h model) plasma collections in six subjects (5 men and 1 woman, age 24 - 64 y, BMI 23.2 - 30.4). Plasma Zn isotope ratios (IR) were measured by inductively coupled plasma - mass spectrometry. After baseline subtraction the plasma Zn IR was divided by the natural Zn IR to give the normalized IR (NIR). The tri-exponential function explained (R^2=0.99) NIR from 0 - 9 days = K_1 exp(-g_1 t) + K_2 exp(-g_2 t) + K_3 exp (-g_3 t), where t is time in days. Since the change of the third term during 24 hours was about 10 %, NIR from 0 - 24 hours was fitted (R^2 = 0.98 -0.99) by the above function

when g_3 was constrained as 0. The estimated coefficients from the 9-d and 24-h kinetic models were similar except for g_2. The sum of three pools as a norm of EZP was calculated from the 0 - 9 day data using the three-pool models (mammillary and catenary; Ramakrishnan. Math. Biosci. 1984;72:373) with a single outlet, which account for the loss of tracer from the system and the quasi-equilibrium between pools. We have shown the mathematical equivalency of the sum of three pools between the mammillary and catenary models, indicating that EZP is invariant by other models. The 24-h spot plasma Zn pool was highly correlated (r^2 0.957) with the sum of three pools, suggesting that the 24-h spot plasma Zn pool is a practical indicator of EZP.

RELATIONSHIP BETWEEN BODY COMPOSITION AND RAPIDLY EXCHANGEABLE ZINC POOL IN HUMANS. N. G. Egger, K .Yokoi, V. M. Sadagopa Ramanujam, H. H. Dayal, N. W. Alcock and H. H. Sandstead. Univ. of Texas Med. Br., Galveston, TX 77555-1109. Kyoto Univ. Grad. Sch. of Med., Kyoto 606-01, Japan.

The rapidly exchangeable zinc (Zn) pool (EZP) is metabolically important. We measured EZP and plasma Zn turnover rate (TR) of humans (5 males and 11 females, normal plasma Zn) after iv administration of ^{67}Zn. Inductively coupled plasma-mass spectrometry was used for measurement of isotope ratios. EZP was estimated from the 24-h "truncated" exponential kinetic model and the 24-h spot plasma Zn pool. TR was calculated as a product of the plasma Zn (i.e., central) compartment size and the initial slope of the disappearance curve. Lean body weight was measured by the bioelectrical impedance. Skeletal muscle mass was estimated from the 24-h urinary creatinine excretion using Wang et al's formula (AJCN 1996;63:863). EZP obtained from the 24-h kinetic model and the 24-h spot plasma Zn pool are identical ($r= 0.994$, $p<0.0001$). Highly significant correlations were found between EZP and body weight ($r=0.925$, $p<0.0001$), lean body weight ($r=0.861$, $p<0.0001$) and 24-h urinary creatinine excretion or skeletal muscle mass ($r=0.900$, $p<0.0001$). In contrast, correlations between EZP and fat mass ($r=0.646$, $p=0.01$) were not so high. These results suggest that the skeletal muscle mass is an important contributor for EZP. TR was correlated well with lean body weight ($r=0.882$, $p<0.0001$) and EZP ($r=0.865$, $p<0.0001$). Because TR (300 - 600 mg/d) is much higher than the excretion rate of Zn (< 10 mg/d), it is closely identical to the Zn uptake rate by tissues. It appears that we observed the same metabolically active mass both initially (TR) and 24 hours later (EZP) through the behavior of the tracer.

SIMPLIFIED PRETREATMENT METHOD FOR PLASMA SAMPLES APPLICABLE TO ZINC KINETIC STUDIES.
V. M. Sadagopa Ramanujam, K. Yokoi, N. G. Egger, H. H. Dayal, N. W. Alcock and H. H. Sandstead. Univ. of TX. Med. Br., Galveston, TX 77555-1109. Kyoto Univ. Grad. Sch. of Med., Kyoto 606-01, Japan.

Common biochemical indicators of zinc (plasma Zn) are insensitive. Prasad et al (JLCM 1963;61:537) utilized ^{65}Zn to show the rapid plasma Zn disappearance in Zn deficiency. Radiation exposure limits its application. Stable Zn isotopes are alternatives. Sample purification is usually required to obtain accurate results for mass spectrometric analysis, but also increases the chance of contamination. We compared two pretreatment methods (extraction vs non-extraction) for Zn isotope ratio (IR) measurement by inductively coupled plasma-mass spectrometry. Plasma samples collected from ten human subjects 5 min to 24 h after iv dose of ^{67}Zn (2 mg) were used for comparison. The plasma (1.5 mL) was digested by hydrogen peroxide (Alcock. BTER 1987;13:363) and dissolved in nitric acid. "Extraction": Zn in the digestate was extracted into CCl_4 as diethylammonium diethyldithiocarbamate chelate followed by back extraction of Zn in nitric acid. The solution was then heated overnight at 80 °C to remove traces of CCl_4, and made up to 10 mL with high purity water after adding yttrium (Y) internal standard. "Non-extraction": Y and high purity water was directly added to the digestate. After subtraction of the baseline the plasma Zn IR was divided by the natural Zn IR to give the normalized IR (NIR). NIR obtained by both the methods agreed well ($^{67}Zn/^{64}Zn$, $r^2=0.997$; $^{67}Zn/^{66}Zn$, $r^2= 0.999$; $^{67}Zn/^{68}Zn$, $r^2=0.999$). Considering the minimum possibility of isobaric interferences in plasma samples, $^{67}Zn/^{68}Zn$ obtained from non-extracted samples is sufficient for routine Zn kinetic analysis.

Principal Investigator: Sandstead, Harold H.
Contract No. DAMD17-95-C-5112

ADDENDUM II

Zinc & Iron Nutriture: Neuropsychological Function of Women

Harold H. Sandstead, M.D. Division of Human Nutrition, Department of Preventive Medicine and Community Health, University of Texas Medical Branch Galveston, TX

This work is being done in collaboration with Nancy W. Alcock, Ph.D., Hari H. Dayal, Ph.D., Norman G. Egger, M.D., and V.M.S. Ramanujam, Ph.D. from our Department, and James G. Penland, Ph.D. of the USDA ARS Human Nutrition Research Center in Grand Forks, ND, and Katsuhiko Yokoi, M.D., Ph.D. of the Department of Social Medicine, University of Kyoto Medical School, Kyoto, Japan.

Our work is in progress. We are testing the hypothesis: zinc and iron repletion will improve neuromotor and cognitive functions of young women. Our study is based on the common occurrence of mild iron and zinc deficiencies among young women, and the essentiality of iron and zinc for cognition.

Due to a decrease in consumption of red meat the average intake of iron and zinc of young US women decreased 40 % from 1977 to 85 (1). This food choice accounts for the median iron (9.8 mg) and zinc (7.4 mg) intakes found by NHANES-II (2), that were 69 & 59 % of the need at 20 % bioavailability (3, 4). Reflecting low iron intakes, the 25th percentile for serum ferritin was 14 μg/L, a level at which bone marrow iron is absent (3).

We found through regression analysis of food frequency data that red meat was one of five predictors of serum ferritin concentration in young women ($n = 38$, $R^2 = 0.53$, $p = 0.0001$) and one of four predictors of zinc status, as indicated by the plasma zinc disappearance constant (k) ($n = 19$, $R^2 = 0.63$, $p = 0.005$) (5).

In our study, zinc status and serum ferritin concentrations were related (5). Serum ferritin was lower when plasma zinc was < 70 μg/dL ($p<0.03$) in 18 subjects in whom the disappearance of injected ^{67}Zn from plasma was measured, and plasma zinc disappearance and plasma zinc turnover were increased when serum ferritin was less than 20 μg/L ($p< 0.05$ and 0.01). When plasma zinc concentration was < 70 μg/dL the disappearance of injected ^{67}Zn was increased ($p< 0.05$). Regression analysis found that serum ferritin concentrations and the 30-60 minute disappearance of injected ^{67}Zn were inversely and non-linearly related ($n = 18$, $R2 = 0.777$, $p< 0.0003$) . The non-linearity was probably caused by an increased intestinal absorption of zinc as iron status decreased (6).

The essentiality of iron for human neuropsychological function was suggested 75 years ago (in retrospect) by findings in children with hookworm (7, 8). More recently iron status was related to cognition of children (9-11), and to EEG power and lateralization, and cognition of young adults (12).

The essentiality of zinc for human cognition was shown by experimental deficiency (13, 14) and repletion (15) (16) studies. A recent double-blind randomized depletion-repletion study of 11 men found abnormal neuromotor, attention, perception, short term visual memory, and spatial functions ($p< 0.05$) after 35 days of depletion when 1, 2, 3, or 4 mg zinc daily per 2500 k calories were fed, as compared to function when 10 mg was fed (14). Consistent with the findings in men, an 8 week double-blind randomized controlled trial of zinc repletion in 17 women with serum ferritin concentrations <20 μg/L found improved ($p< 0.004$) short term visual memory (17) in 11 subjects given 30 mg zinc daily plus selected micronutrients and no improvement in 6 women given micronutrients alone (15). A recent 10 week double-blind randomized controlled repletion trial in urban first graders (6-9 years) from Chongqing, Qingdao and Shanghai, China found that zinc repletion with or without selected micronutrients improved key tapping, circular

tracking, matching complex designs, visual memory of complex designs, and concept formation measured by recognition of oddity, while micronutrients alone did not ($p < 0.05$) (16).

The study we are doing that is of relevance to military women on young women is a 16 week double blind randomized controlled repletion trial, with a cross-over at 8 weeks, of 30 mg iron or 30 mg zinc daily and/or selected micronutrients alone on neuropsychological functions of 60 non-anemic women, ages 19-40 years, who have serum ferritin concentrations from 5-18 µg/L. Zinc status is characterized by ^{67}Zn kinetics and white blood cell zinc concentrations. Twenty women with serum ferritin >30µg/L serve as normal controls. Measurements of neuropsychological functions are done using a computerized task battery developed by James G. Penland, Ph.D. of the USDA ARS Human Nutrition Research Center in Grand Forks, ND. In two years we will know the outcome.

Our study has important implications for military women. Job performance often requires optimal neuropsychological function. If our hypothesis is proven true dietary recommendations will need to be revised to assure that intakes of bioavailable iron and zinc are sufficient to meet the needs of women. On a broader scale, our study has implications for all persons at risk of iron and zinc deficiencies. If we find that neuropsychological functions are improved by iron and/or zinc repletion, dietary guidelines and feeding programs for groups at risk will need to be revised to assure that intakes of bioavailable iron and zinc are adequate. Research will be needed to learn how iron and zinc affect human cognition throughout the life cycle.

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The University of Texas Medical Branch at Galveston

Division of Human Nutrition,
Department of Preventive Medicine and Community Health

April 30, 1997

Commander
US Army Research Institute of Environmental Medicine
Attn: MCMR-UE-RP/ Ms. Marie E. Stevens
Natick, MA 01760-5007

Subject: Contract No. DAMD 17-95-C-5112 Quarterly Report

Dear Ms. Stevens

Attached is the Quarterly Report for December 22, 1996 to March 22, 1997 for Contract No. DAMD 17-95-C-5112. I apologize for the delay in submitting this report. Subject recruitment has increased this quarter and we have been very busy. If you have questions or need further information, please contact me at 409 772-4661.

Thank you,

Harold H. Sandstead, MD
Professor

Attachment

Department of Preventive Medicine and Community Health
Division of Human Nutrition
The University of Texas Medical Branch, 700 Harborside Dr.
Galveston, Texas 77555-1109
Phone: 409-772-4661 FAX: 409-772-6287

Principal Investigator: Sandstead, Harold H.
Contract No. DAMD17-95-C-5112

QUARTERLY REPORT

1. Contract No.: DAMD17-95-C-5112 2. Report Date: 4-30-97
3. Reporting Period from: 12-22-96 to 3-22-97
4. PI: Harold H. Sandstead 5. Telephone No. (409) 772-4661
6. Institution: The University of Texas Medical Branch
7. Project Title: Repletion of Zinc and Iron deficiencies improves cognition of premenopausal women.
8. Current Staff, with percent effort of each on project:

Harold H. Sandstead	15 %	Nancy W. Alcock	10 %
VM Sadagopa Ramanujam	25 %	Hari H. Dayal	10 %
Norman G. Egger	100 %	Virginia Rehman	50 %
Jackie Curtis	75 %	Michael Vega	75 %

10. Administrative and logistical matters.

a. This quarter we improved recruitment. Sixty two women contacted us by phone to learn about the study. Thus a total of 247 women have expressed interest since the start of the study. Eighteen respondents met the selection criteria and were screened during an outpatient visit to the Clinical Research Center (CRC). Thus 75 respondents have undergone detailed screening since the start of the study.

b. Data collected during neuropsychological performance assessment have been sent to our collaborator James G. Penland at the USDA ARS Grand Forks Human Nutrition Research Center for analysis. Blood and urine samples for various other assays have been sent to collaborators at this and other institutions.

c. Enrollment of subjects improved this quarter, and more subjects with low serum ferritin levels were identified. We have continued to advertise in newspapers, local health clubs, and on campuses of local and regional Universities. We continue to focus on vegetarians (our current flyer is attached as an addendum II).

d. We were visited by Mrs. C. Smith, US Army Medical Research and Material Command, and Mrs. J. LeSage, Clinical Investigator Regulatory Office on March 6, 1997 for an audit of our procedures concerning human use. A follow-up letter from Mrs. Smith indicated our procedures were found satisfactory.

e. The subject consent form was changed to include the option of doing the study as an outpatient (enclosed is the current form approved on April 1, 1997). Mrs. Smith suggested we change the order of reimbursement so that a larger proportion of the payment will be given at the end of the study (see letter March 21, 1997, from Mrs. Claudia Bartz, Colonel, Deputy Chief of Staff for Regulatory Compliance & Quality). A request for approval of such a change has been sent to the UTMB Institutional Review Board.

f. The administrative assistant accepted a job elsewhere. She has been replaced. The laboratory technician will move to another city. A search for his replacement has been initiated.

g. The research group continues to meet weekly.

11. Scientific progress for the quarter in terms of the tasks or objectives listed in the statement of work for this contract. Explain deviations where this isn't possible. Include data where possible.

a. Nine subjects completed the assessment of zinc status by measurement of zinc kinetics Thus zinc kinetic data have been compiled on 29 subjects and 6 male controls have been studied since the beginning of the study. This quarter six

subjects completed the baseline assessment of neuropsychological performance and began the experimental treatment. Thus 14 subjects have progressed this far since the beginning of the study. This quarter 7 subjects completed the first 8 weeks of treatment and were switched to their other treatment. Nine subjects have progressed this far since the beginning of the study. Three subjects have completed the entire study.

b. The 3 posters submitted to "Experimental Biology '97", April 6-9, 1997, in New Orleans, LA were presented. The abstracts were attached to our previous report and are attached here as well (Addendum I).

c. Analysis of white blood cell zinc: White blood cells and platelets were isolated from 8 subjects this quarter. The yield for the fractions was:

Lymphocytes (1.29 - 6.77) x 10^3 /μL suspension
Granulocytes (1.47 - 16.10) x 10^3 /μL suspension
Platelets (514 - 2179) x 10^3 /μL suspension

The percent yield of the lymphocytes isolated was 69.0 - 92.4 in 7 of the 8 subjects. The exception had 57 percent "lymphocytes", but 26.9 percent monocytes were counted in this fraction. From 6 of the 8 subjects the isolated granulocyte fraction were 90.9 - 96.6 percent pure.

The zinc content of the cell fractions was determined for 5 of the 8 subjects. Extracts of the cells from the other 3 subjects were frozen for later assay. With the exception of the subject with the highest platelet contamination in the lymphocyte fraction, where the zinc concentration was elevated, the concentrations of zinc in all cell fractions from the 5 subjects were within the reported reference range.

d. Analysis of plasma beta-hydroxybutyrate: Specimens for this assay were stored for future analysis because a new batch of the commercial enzyme kit used was found to have deteriorated. Therefore a manual method with different sources for the reagents will replace the kit method. Standardization will be established with quality control serum.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

a. Continue recruiting at the present pace.

b. Prepare three reports based on our presentations at EB '97.

Principal Investigator: Sandstead, Harold H.
Contract No. DAMD17-95-C-5112

ADDENDUM I

Abstracts presented at Experimental Biology, '97, April 6-9, 1997 New Orleans, Louisiana.

MATHEMATICAL MODELS FOR TWENTY-FOUR-HOUR AND NINE-DAY ZINC KINETICS IN HUMANS. K. Yokoi, V. M. Sadagopa Ramanujam, N. G. Egger, H. H. Dayal, N. W. Alcock and H. H. Sandstead. Univ. of Texas Med. Br., Galveston, TX 77555-1109. Kyoto Univ. Grad. Sch. of Med., Kyoto 606-01, Japan.

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The University of Texas Medical Branch at Galveston

School of Medicine
Graduate School of Biomedical Sciences
School of Allied Health Sciences
School of Nursing

Marine Biomedical Institute
Institute for the Medical Humanities
UTMB Hospitals and Clinics

Research Administrative Services

April 1, 1997

MEMORANDUM

TO: Harold H. Sandstead, MD
Department of Preventive Medicine and Community Health 1009

FROM: Wayne R. Patterson, Ph.D.
Director of Institutional Review Coordination
Research Administrative Services 0136

SUBJECT: IRB #94-295 - Administrative Approval of a Protocol Modification and a Revised Subject Consent Form.
"Effect of Zinc and Iron Repletion on Cognition of Premenopausal Women with Mild Zinc and Iron Deficiencies"

The Institutional Review Board acknowledges receipt of your memorandum dated 3/27/97 requesting approval of a protocol modification and a revised subject consent form. The modification includes the treatment interval of Phase 3 and 4 of the study is 8 weeks instead of 4 weeks. The subject consent form has been revised to reflect this change. The protocol modification and revised subject consent form were reviewed and administratively approved by Dr. Frank C. Schmalstieg, Jr., IRB Chairman on 4/1/97. You may now proceed with your research project.

Attached is the revised subject consent form with the date of the IRB approval. Please use this copy of the consent form with the IRB approval date and make additional copies as they are needed.

WRP/as

Attachment: Revised Subject Consent Form

xc: Clinical Research Center 0331

3.114 ADMINISRATION BUILDING 301 UNIVERSITY BOULEVARD GALVESTON, TEXAS 77555-0136
(409) 772-3482 FAX (409) 772-4195

Subject Consent Form

I have been asked to participate as a subject in the research project entitled "The effect of zinc and iron repletion on cognition of mildly zinc and iron deficient premenopausal women" under the direction of Harold H. Sandstead, M.D. This project will be conducted at the University of Texas Medical Branch, 301 University Blvd, Galveston, TX 77555.

PURPOSE OF THE STUDY

I understand that the purpose of the study is to determine if correction of low zinc and iron status will improve memory and attention, and other functions that require zinc and iron.

REQUIREMENTS FOR PARTICIPATION

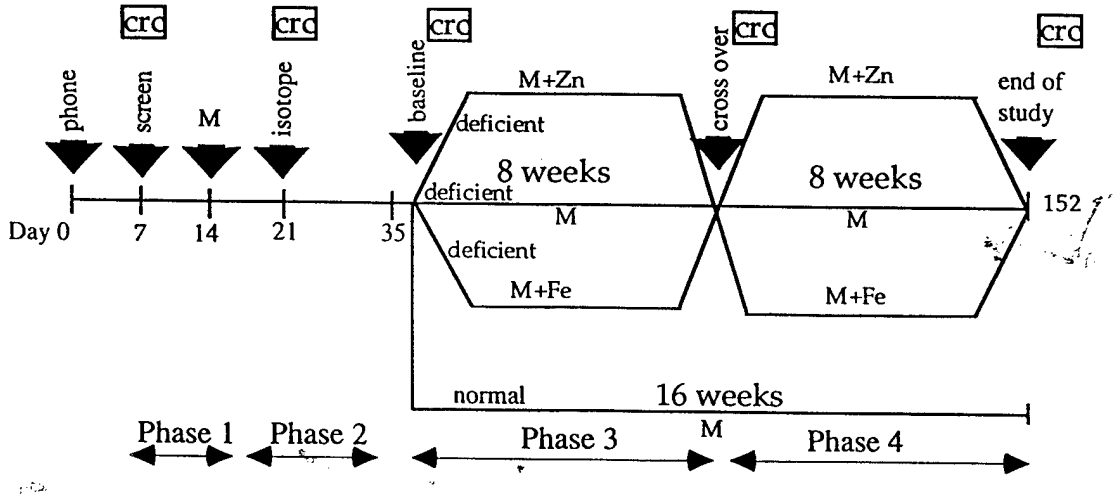
Good health, moderate habits, not recently taking nutrition supplements and understanding this protocol are requirements for participation on this study. It is essential that subjects are not pregnant and have regular menses.

While I will not be tested for pregnancy, I understand that failure to menstruate "on time" will be a disqualifying criterion. It is therefore essential that I do not volunteer to participate if I think I am pregnant, plan to become pregnant or am unwilling to prevent pregnancy while I participate in the study.

PROCEDURES

The following diagram is intended to improve my understanding of this study.

CRC=Clinical Research Center
M=Micronutrients
M+Fe=Micronutrients plus Iron
M+Zn=Micronutrients plus Zinc



Phase 1: This Phase involves 150 individuals who are screened for health and nutrition status by questionnaires, and chemical measurements of blood and urine. This will require about 45 minutes. About 30 mL of blood (about 4 tablespoons) will be drawn from a vein and a urine sample will be collected. Their medical and dietary histories will be reviewed and they will be

Initial Date

given a physical examination. Individuals who participate in screening will be given a copy of their Consent Statement and $25.

Phase 2: One hundred individuals from phase 1, who meet the study criteria will be invited to participate in this phase. They will be started on a daily vitamin-mineral supplement which will be given for at least seven days before admission to The University of Texas Medical Branch Clinical Research Center (CRC). They will be given the choice to be admitted to the CRC on the 10th day of their menstrual cycle before 3:00 PM for 2 nights or come as an outpatient. The diet they are fed will be low in zinc. About 300 hairs will be removed by scissors from the lower back of their scalp for analysis of hair zinc. The first night they will not eat between 6:00 PM and about 10:00 AM the next morning. At about 6:45 AM the next morning small tubes will be placed in the large veins in the bend of each elbow. These veins are commonly used for taking blood samples. Fluid (saline) will be slowly administered through one of the tubes, the other will be plugged with a stopper. About 30 minutes after placement of the tubes the subject will empty her bladder and begin collection of all urine for the next 24 hours. A 15 mL (about 2 tablespoons) blood sample will then be taken and 2 mg (the weight of a few grains of salt) of a rare form of zinc will be injected. After 5, 15, 30, 40, 50, 60, 90 and 120 minutes, 7.5-15 mL (about 1-2 tablespoons) blood samples will be taken. Subjects will then be fed. Twenty four hours after injection, 20 mL blood samples will be taken. In the afternoon the subject will be introduced to the computerized tasks for measuring brain function. The tasks will be somewhat similar to a computer game. The test-giver will explain the tasks and show the subject how to do them. The subject will then do a trial run to become comfortable with the procedure. This learning process will take about 45-60 minutes. The next morning the 24 hour urine collection will end at about 7:15 AM and the 24 hour blood sample will be taken. The total amount of blood drawn will be less than 180 mL (about 24 tablespoons). The subject will then be discharged with a supply of the daily vitamin-mineral supplement which they will continue to take. Subjects who complete the second phase will be paid $150.

Phase 3: Eighty subjects who completed Phase 1 or both phase 1 and 2, and who meet the study criteria will be invited to participate. They will be given the choice to be admitted to the CRC or come as an outpatient on the 10th day of their menstrual cycle. Subjects will be asked not to drink alcohol or take any medications including aspirin, ibuprofen, acetaminophen at least for 48 hours preceding admission. During this admission baseline measurements will be made of memory and other brain functions, and of other functions that are likely to be affected by zinc and iron repletion.

Subjects will be admitted to the CRC in the morning for a 2 night stay or come as an outpatient. Tests will include measurements of brain function by computer, measurements of the strength of contraction of a thumb muscle, measurement of fat mass, measurement of taste acuity, measurement of energy metabolism by a breathing test, measurement of the function of certain zinc and iron dependent enzymes, measurements of the concentrations of zinc and iron in components of blood, and measurements of substances in the blood such as vitamins, hormones, and amino acids that can be affected by zinc and iron status.

Brain functions will be measured by computerized tasks in the morning, and will require about 90 minutes.

The muscle function tests involves electrical stimulation of a nerve at the wrist, causing minor discomfort. The strength of contraction of a thumb muscle will be measured.

During each admission, the fat mass of the subjects will be determined by bioelectrical impedance by applying an undetectable current to the body. This is a safe, simple and quick method.

The taste test involves placement of a small filter paper containing a flavor to the subject's tongue followed by stimulation with a low voltage electric current that will be difficult for the subject to detect.

The morning after admission a 'breathing test' will be done to measure the subject's oxygen consumption and carbon dioxide production. The subject will breath oxygen exhale through a mouth piece while a clip is placed on the subjects nose to prevent leakage. The test will be done early in the morning, about 6:00 AM while the subject is at rest.

Soon after completion of the breathing test the subjects will be given one tablet of 500 mg chlorzoxazone, a muscle relaxant, to measure function of certain enzymes that require iron and zinc. A blood sample will be taken two hours later. After this test subjects will be fed breakfast.

After completion of the above the subject will be assigned one of three vitamin-mineral treatments. One treatment will contain vitamins and minerals, one treatment will contain vitamins and minerals with iron, and one treatment will contain vitamins and minerals with zinc. Neither the subject nor the investigators will know which treatment the subject is receiving until the study is finished. It is essential to keep the treatments secret to avoid bias in interpretation of results.

At discharge the subject will be given a bottle of 35 tablets containing the treatment. The treatment will be taken early in the morning. The subject will be contacted weekly by phone to determine progress.

After **8** weeks the subject's brain functions and the above indices affected by zinc and iron nutrition will be tested again on the 10th-12th day of the subject's menstrual cycle. After these tests the subject will be given $150 and a new supply of tablets containing essential vitamins and minerals.

Phase 4: Phase 4 is a continuation of phase 3. Subjects will continue to take the vitamin-mineral tablets. After **8** weeks of treatment the subject will be readmitted to the CRC on the 10th day of her menstrual cycle for repeat measurements of the tests done in phase 3. After the tests are completed the subject will be given $100 and a copy of her medical record.

When the study is completed and results published the subject will be mailed a copy of the report.

NUMBER OF SUBJECTS PARTICIPATING

150 subjects will participate in the initial screening; 100 subjects will participate in phase 2 ; and 80 subjects will participate in phases 3 and 4.

RISKS OF PARTICIPATION

I understand that all research entails some risk. Risks of this study are very small and similar to those associated with going to the doctor to be evaluated and receive routine care. Physical risks are those associated with drawing blood from a large vein in the arm, being given a small intravenous dose of a non-toxic zinc tracer, and of taking a small one tablet dose of a common muscle relaxant, chlorzoxazone.

To keep the risks very small the blood drawing will be done by a highly experienced professional who draws blood from adults almost daily. The total amount of blood taken during the study will be less than 400 mL (about 14 oz), a relatively small amount for an adult, and an amount that is substantially less than the amount given during blood donation. Rarely the process of drawing blood causes pain or bruising. Infection is very rare.

The administration of the zinc tracer to humans involves very little risk. Zinc is an essential nutrient. About 4 % of zinc in nature occurs in the form zinc that is used in this project as a tracer. The tracer is non-radioactive. It is handled in the body like all other zinc. Two milligrams of the tracer is dissolved in a very dilute salt solution and given intravenously. This amount is similar to the zinc requirement of an adult for one day. This amount is a common component of intravenous nutrition preparations that are used every day in hospitals. The tracer will not cause fever or infections.

Chlorzoxazone is a muscle relaxant medication that is used by doctors to treat painful muscles. The usual dose is 1500 mg to 3000 mg daily. The amount of chlorzoxazone administered in this project, 500 mg, will be 1/3 of the therapeutic dose. Infrequent side effects of the therapeutic dose include gastrointestinal distress, drowsiness, dizziness, lightheadedness, and gastrointestinal bleeding. The amount given to the subjects in this project are unlikely to cause these effects. Precautions will exclude subjects in the study who have known intolerance to chlorzoxazone or who are taking central nervous system depressants.

There are no obvious psychological or social risks associated with this project. Confidentiality will be maintained by keeping records in a locked file. Health records will be in the subjects hospital chart.

The data obtained from this study will be used for research purposes here at the University of Texas Medical Branch, Galveston, Texas, and with other collaborators at the United States Department of Agriculture (USDA), Grand Forks, North Dakota and at the Department of Social and Preventive Medicine - Kyoto University - Graduate School of Medicine, Kyoto, Japan. We will maintain total confidentiality by numbering subjects so that others cannot identify them.

BENEFITS TO THE SUBJECT

I understand that the direct benefits to me include a medical screening evaluation, and the possibility that any nutritional deficiencies I might have will be temporarily corrected.

REMOVAL FROM THE STUDY

I understand that I may be removed from this study at the Principle Investigators discretion.

ALTERNATIVE TREATMENT

N/A. There is no alternative procedure/treatment.

REIMBURSEMENT FOR EXPENSES

I understand that I will be reimbursed for lost time, travel, parking, meals, etc. in the amount of $25 when completing phase 1, $150 when completing phase 2, $150 when completing phase 3, and $100 when completing phase 4.

COSTS OF PARTICIPATION

I understand the study will involve my time and two nights stay in the CRC on four occasions. There will be some discomfort with placement of tubes in veins, and the muscle function testing but this is minimal.

UTMB STANDARD CLAUSES

1. I understand that informed consent is required of all persons in this project.

2. The principal and alternate procedures, including the experimental procedures in this project, have been identified and explained to me in language that I can understand.

3. The risks and discomforts from the procedures have been explained to me.

4. The expected benefits from the procedures have been explained to me.

Initial Date

5. An offer has been made to answer any questions that I may have about these procedures. If I have any questions before, during or after the study, I may contact Dr. Harold H. Sandstead at (409) 772-4661.

6. I have been told that I may refuse to participate or stop my participation in this project at any time without prejudice and without jeopardizing my medical care at UTMB. All new findings during the course of this research which may influence my desire to continue or not to continue to participate in this study will be provided to me as such information becomes available.

7. I have been told that The University of Texas Medical Branch at Galveston, like virtually all other Universities in the United States, does not have a mechanism for compensation of the injured research subject. Therefore, I understand that I cannot look to any such mechanism to receive financial remuneration for any such injuries resulting from my participation in this project.

   I understand that I am authorized all necessary medical care for injury or illness which is the proximate result of my participation in this research. UTMB must provide such medical care when conducting research on private citizens. Other than medical care that may be provided (and any other remuneration specifically stated in this consent form), there is no other compensation available for my participation in this research study; however, I understand this is not a waiver or release of my legal rights.

8. If I have any questions regarding my rights as a patient participating in this study or research-related injury, I may contact Wayne R. Patterson in Research Administration Services at (409) 772-3482.

9. I have a right to privacy, and all information that is obtained in connection with this study and that can be identified with me will remain confidential as far as possible within state and federal law. However, information gained from this study that can be identified with me may be released to no one other than the investigators, research collaborators and my physician. The results of this study may be published in scientific journals without identifying me by name.

10. It is the policy of the U.S. Army Medical Research and Material Command that data sheets are to be completed on all volunteers participating in research for entry into this Command's Volunteer Registry Data Base. The information to be entered into this confidential data base includes you name, address, Social Security number, study name and dates. The intent of the data base is two-fold: first, to readily answer questions concerning an individual's participation in research sponsored by USAMRMC; and second, to ensure that the USAMRMC can exercise its obligation to ensure research volunteers are adequately warned (duty to warn) of risks and to provide new information as it becomes available. The information will be stored at USAMRMC for a minimum of 75 years.

11. Representatives of the U.S. Army Medical Research and Material Command are eligible to review research records as a part of their responsibility to protect human subjects in research.

I voluntarily agree to participate as a subject in the above named project. I understand that I will be given a copy of the consent form I have signed.

[redacted] Initial Date

Date _____ Signature of Subject _____

Type/Print Name of Subject _____

Type/Print permanent address of Subject _____

Date _____ Signature of Witness _____

Type/Print Name of Witness _____

Type/Print permanent address of Witness _____

Using language that is understandable and appropriate I have discussed this project and the items listed above with the subject and/or his authorized representatives.

Date _____ Signature of Project Director or his/her Representative _____

Zinc-Iron study: Principle Investigator: Sandstead, Harold H.

[redacted]

Initial _____ Date _____

Principle Investigator: Sandstead, Harold H.
Contract No. DAMD17-95-C-5112

ADDENDUM II

WOMEN NEEDED

TO MEASURE THE EFFECT OF IRON & ZINC ON MEMORY

VEGETARIANS PREFERRED

REIMBURSEMENT UP TO $425

AGES 19-40

The University of Texas Medical Branch at Galveston

Division of Human Nutrition,
Department of Preventive Medicine and Community Health

July 16, 1997

Commander
US Army Research Institute of Environmental Medicine
Attn: MCMR-UE-RP/Ms. Marie E. Stevens
Natick, MA 01760-5007

Subject: Contract No. DAMD 17-95-C-5112 Quarterly Report

Dear Ms. Stevens:

Attached is the Quarterly Report for March 23 - June 22 for Contract No. DAMD 17-95-C-5112. If you have questions or need further information, please contact me at 409-772-4661.

Thank you.

Sincerely,

Harold H. Sandstead, MD
Professor

Enclosures:

IRB Approval Letter and Subject Consent Form - April 25, 1997

Manuscripts:

Comparison of Tri-Exponential and Truncated Models in Plasma Zinc (Zn) Kinetics

Simplified Pretreatment Method for the Analysis of Plasma Samples Applicable to Zinc Kinetic Studies

Department of Preventive Medicine & Community Health
Division of Human Nutrition
700 Harborside Drive, Ewing Hall 3.102
Galveston, Texas 77555-1109
409-772-4661 FAX: 409-772-6287

Principal Investigator: Sandstead, Harold H.
Contract No. DAMD17-95-C-5112

QUARTERLY REPORT

1. Contract No.: DAMD17-95-C-5112 2. Report Date: 1-30-97
3. Reporting Period from 3/23/97 to 6/22/97
4. PI: Harold H. Sandstead 5. Telephone No. (409) 772-4661
6. Institution: The University of Texas Medical Branch
7. Project Title: Repletion of Zinc and Iron deficiencies improves cognition of premenopausal women.
8. Current Staff, with percent effort of each on project:

Harold H. Sandstead	15 %	Nancy W. Alcock	10 %
VM Sadagopa Ramanujam	25 %	Hari H. Dayal	10 %
Norman G. Egger	90 %	Jackie Curtis	75 %
Michael Loftus	100 %		

Principal Investigator: Sandstead, Harold H.
Contract No. DAMD17-95-C-5112

10. Administrative and logistical matters.

a. This quarter recruitment continued to improve. Seventy five women responded to our advertisements (322 since the start of the study). Of these 37 met the criteria for outpatient screening (118 since the start of the study).

b. Data collected from the neuropsychological assessment are being stored on computer disks until they can be sent to our Co-Investigator James G. Penland, Ph.D. at the USDA ARS Grand Forks Human Nutrition Research Center in Grand forks, ND for analysis. The center was severely damaged this spring by flood. It is anticipated the Center will resume full operation in November or December, 1997.

c. Blood and urine samples for various "add-on" assays by collaborators have been sent to them. With regard to measurements of neurotransmitters and opioids in blood, the research program of our collaborator at the USDA ARS Human Nutrition Research Center in Beltsville, MD was discontinued. Therefore those assays will not be done.

d. While enrollment improved we are unable to identify why. We will continue to advertise in newspapers, at local health clubs, and local and regional Universities.

e. We continue to have a problem getting subjects to complete the study. Some have not completed because of relocation. Others have simply lost interest. or interruptions. To improve motivation we are keeping in closer touch by frequent phone calls and more visits to the laboratory. In addition, as suggested by Mrs. Smith, who audited us earlier this year, we have increased the last payment and decreased interim payments. This action was approved by the Institutional Review Board (March 21, 1997 letter, from Mrs. Claudia Bartz, Colonel, Deputy Chief of Staff for Regulatory Compliance & Quality). The subject consent form was approved by the UTMB Institutional Review Board, (April 25, 1997 letter).

f. The laboratory technician has been replaced.

g. The research group continues to meet weekly.

11. Scientific progress for the quarter in terms of the tasks or objectives listed in the statement of work for this contract. Explain deviations where this isn't possible. Include data where possible.

a. Sixteen subjects completed the assessment of zinc kinetics (42 since the start of the study). Twelve subjects progressed to the treatment trial (26 since the start of the study). Four subjects completed the first 8 weeks of treatment and the cross over to the second 8 weeks of treatment (13 since the start of the study). Three subjects completed the study (7 since the start of the study). As noted above it has proved difficult to keep subjects for the duration of the study. In addition, we are seriously behind schedule because of our earlier difficulties with recruiting.

b. Two manuscripts are nearly completed and ready for submission to peer review (see addendum).

c. A poster will be presented at the American Society of Clinical Nutrition 37th annual meeting, July 24-26,1997 in Montreal, Canada (see addendum).

d. Since the previous report, the white cell isolation procedure has been performed on 15 subjects. Although performed by a different technical assistant, there was no difficulty with the procedure. In 4 individuals, the granulocyte count has been very low, perhaps due to some clumping of the cells, and therefore an incomplete sample obtained for counting and zinc determination. Discussion with Dr. Beck whose published procedure is used, indicated that this is a sporadic finding, the reason for which has not been identified. A critical examination of the various steps for loss of white cells is being pursued. In other subjects the granulocyte count was similar to previously reported, and the purity of the cell fractions comparable.

The zinc analysis on the various cell fractions is pending, as this is performed in batches, in order to contain the ever present potential for environmental contamination. The use of an electrodeless discharge zinc lamp, has improved baseline drift associated with the hollow cathode lamp in the past.

It is planned to measure magnesium in the cell fractions, to check on the constancy of this, and the possibility of using this as a reference value.

e. Difficulty with serum beta-hydroxy-butyrate kits from Sigma Chemical Co. has been resolved, and none of the 24 specimens stored at -70° C had an elevated level.

12. Use additional page(s) to present a brief statement of plans or milestones for the next quarter.

a. Continue recruiting at the present pace.

b. Prepare manuscript reporting relationships between zinc kinetics and body composition in 40 subjects.

ADDENDUM

The University of Texas Medical Branch at Galveston

School of Medicine
Graduate School of Biomedical Sciences
School of Allied Health Sciences
School of Nursing

Marine Biomedical Institute
Institute for the Medical Humanities
UTMB Hospitals and Clinics

Research Administrative Services

April 25, 1997

MEMORANDUM

TO: Harold H. Sandstead, MD
Department of Preventive Medicine and Community Health 1009

FROM: Wayne R. Patterson, Ph.D.
Director of Institutional Review Coordination
Research Administrative Services 0136

SUBJECT: IRB #94-295 - Administrative Approval of a Revised Subject Consent Form.
"Effect of Zinc and Iron Repletion on Cognition of Premenopausal Women with Mild Zinc and Iron Deficiencies"

The Institutional Review Board acknowledges receipt of your memorandum dated 4/21/97 requesting approval of a a revised subject consent form. The reimbursement section has been revised as follows: 1) Page 2, second paragraph, last sentence, subjects who complete the second phase has been changed from $150.00 to $100. 2) Page 3, fifth paragraph, last sentence, after these tests the subject will be given $150.00 has been changed to $125.00, and a new supply of tablets containing essential vitamins and minerals. 3) Page 3, sixth paragraph, last sentence, after these tests are completed the subject will be given $100.00 has been changed to $175.00 and a copy of her medical record. 4) Page 4, seventh paragraph, $150.00 when completing phase 2 has been changed to $100.00, $150.00 when completing phase 3 has been changed to $125.00 and $100.00 when completing phase 4 has been changed to 175.00. This request was reviewed and administratively approved by Dr. Frank C. Schmalstieg, Jr., IRB Chairman on 4/25/97. You may now proceed with your research project.

Attached is the revised subject consent form with the date of the IRB approval. Please use this copy of the consent form with the IRB approval date and make additional copies as they are needed.

WRP/as

Attachment: Revised Subject Consent Form

xc: Clinical Research Center 0331

3.114 ADMINISTRATION BUILDING 301 UNIVERSITY BOULEVARD GALVESTON, TEXAS 77555-0136
(409) 772-1582 FAX (409) 747-0044

Subject Consent Form

IRB APR 25 1997

I have been asked to participate as a subject in the research project entitled "The effect of zinc and iron repletion on cognition of mildly zinc and iron deficient premenopausal women" under the direction of Harold H. Sandstead, M.D. This project will be conducted at the University of Texas Medical Branch, 301 University Blvd, Galveston, TX 77555.

PURPOSE OF THE STUDY

I understand that the purpose of the study is to determine if correction of low zinc and iron status will improve memory and attention, and other functions that require zinc and iron.

REQUIREMENTS FOR PARTICIPATION

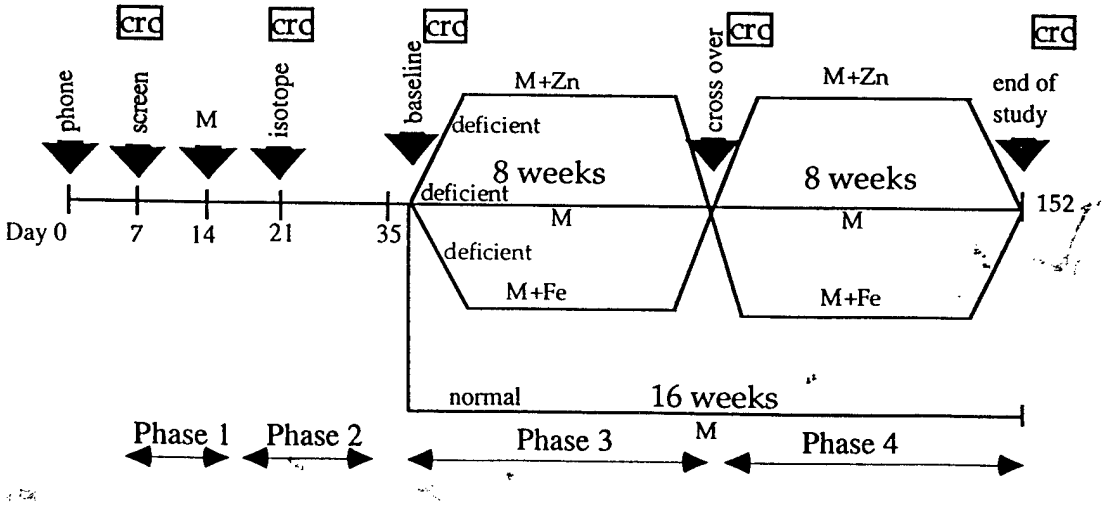
Good health, moderate habits, not recently taking nutrition supplements and understanding this protocol are requirements for participation on this study. It is essential that subjects are not pregnant and have regular menses.

While I will not be tested for pregnancy, I understand that failure to menstruate "on time" will be a disqualifying criterion. It is therefore essential that I do not volunteer to participate if I think I am pregnant, plan to become pregnant or am unwilling to prevent pregnancy while I participate in the study.

PROCEDURES

The following diagram is intended to improve my understanding of this study.

CRC=Clinical Research Center
M=Micronutrients
M+Fe=Micronutrients plus Iron
M+Zn=Micronutrients plus Zinc



Phase 1: This Phase involves 150 individuals who are screened for health and nutrition status by questionnaires, and chemical measurements of blood and urine. This will require about 45 minutes. About 30 mL of blood (about 4 tablespoons) will be drawn from a vein and a urine sample will be collected. Their medical and dietary histories will be reviewed and they will be

Initial Date

given a physical examination. Individuals who participate in screening will be given a copy of their Consent Statement and $25.

Phase 2: One hundred individuals from phase 1, who meet the study criteria will be invited to participate in this phase. They will be started on a daily vitamin-mineral supplement which will be given for at least seven days before admission to The University of Texas Medical Branch Clinical Research Center (CRC). They will be given the choice to be admitted to the CRC on the 10th day of their menstrual cycle before 3:00 PM for 2 nights or come as an outpatient. The diet they are fed will be low in zinc. About 300 hairs will be removed by scissors from the lower back of their scalp for analysis of hair zinc. The first night they will not eat between 6:00 PM and about 10:00 AM the next morning. At about 6:45 AM the next morning small tubes will be placed in the large veins in the bend of each elbow. These veins are commonly used for taking blood samples. Fluid (saline) will be slowly administered through one of the tubes, the other will be plugged with a stopper. About 30 minutes after placement of the tubes the subject will empty her bladder and begin collection of all urine for the next 24 hours. A 15 mL (about 2 tablespoons) blood sample will then be taken and 2 mg (the weight of a few grains of salt) of a rare form of zinc will be injected. After 5, 15, 30, 40, 50, 60, 90 and 120 minutes, 7.5-15 mL (about 1-2 tablespoons) blood samples will be taken. Subjects will then be fed. Twenty four hours after injection, 20 mL blood samples will be taken. In the afternoon the subject will be introduced to the computerized tasks for measuring brain function. The tasks will be somewhat similar to a computer game. The test-giver will explain the tasks and show the subject how to do them. The subject will then do a trial run to become comfortable with the procedure. This learning process will take about 45-60 minutes. The next morning the 24 hour urine collection will end at about 7:15 AM and the 24 hour blood sample will be taken. The total amount of blood drawn will be less than 180 mL (about 24 tablespoons). The subject will then be discharged with a supply of the daily vitamin-mineral supplement which they will continue to take. Subjects who complete the second phase will be paid $100.

Phase 3: Eighty subjects who completed Phase 1 or both phase 1 and 2, and who meet the study criteria will be invited to participate. They will be given the choice to be admitted to the CRC or come as an outpatient on the 10th day of their menstrual cycle. Subjects will be asked not to drink alcohol or take any medications including aspirin, ibuprofen, acetaminophen at least for 48 hours preceding admission. During this admission baseline measurements will be made of memory and other brain functions, and of other functions that are likely to be affected by zinc and iron repletion.

Subjects will be admitted to the CRC in the morning for a 2 night stay or come as an outpatient. Tests will include measurements of brain function by computer, measurements of the strength of contraction of a thumb muscle, measurement of fat mass, measurement of taste acuity, measurement of energy metabolism by a breathing test, measurement of the function of certain zinc and iron dependent enzymes, measurements of the concentrations of zinc and iron in components of blood, and measurements of substances in the blood such as vitamins, hormones, and amino acids that can be affected by zinc and iron status.

Brain functions will be measured by computerized tasks in the morning, and will require about 90 minutes.

The muscle function tests involves electrical stimulation of a nerve at the wrist, causing minor discomfort. The strength of contraction of a thumb muscle will be measured.

During each admission, the fat mass of the subjects will be determined by bioelectrical impedance by applying an undetectable current to the body. This is a safe, simple and quick method.

The taste test involves placement of a small filter paper containing a flavor to the subject's tongue followed by stimulation with a low voltage electric current that will be difficult for the subject to detect.

The morning after admission a 'breathing test' will be done to measure the subject's oxygen consumption and carbon dioxide production. The subject will breath oxygen exhale through a mouth piece while a clip is placed on the subjects nose to prevent leakage. The test will be done early in the morning, about 6:00 AM while the subject is at rest.

Soon after completion of the breathing test the subjects will be given one tablet of 500 mg chlorzoxazone, a muscle relaxant, to measure function of certain enzymes that require iron and zinc. A blood sample will be taken two hours later. After this test subjects will be fed breakfast.

After completion of the above the subject will be assigned one of three vitamin-mineral treatments. One treatment will contain vitamins and minerals, one treatment will contain vitamins and minerals with iron, and one treatment will contain vitamins and minerals with zinc. Neither the subject nor the investigators will know which treatment the subject is receiving until the study is finished. It is essential to keep the treatments secret to avoid bias in interpretation of results.

At discharge the subject will be given a bottle of 35 tablets containing the treatment. The treatment will be taken early in the morning. The subject will be contacted weekly by phone to determine progress.

After 8 weeks the subject's brain functions and the above indices affected by zinc and iron nutrition will be tested again on the 10th-12th day of the subject's menstrual cycle. After these tests the subject will be given $125 and a new supply of tablets containing essential vitamins and minerals.

Phase 4: Phase 4 is a continuation of phase 3. Subjects will continue to take the vitamin-mineral tablets. After 8 weeks of treatment the subject will be readmitted to the CRC on the 10th day of her menstrual cycle for repeat measurements of the tests done in phase 3. After the tests are completed the subject will be given $175 and a copy of her medical record.

When the study is completed and results published the subject will be mailed a copy of the report.

NUMBER OF SUBJECTS PARTICIPATING

150 subjects will participate in the initial screening; 100 subjects will participate in phase 2 ; and 80 subjects will participate in phases 3 and 4.

RISKS OF PARTICIPATION

I understand that all research entails some risk. Risks of this study are very small and similar to those associated with going to the doctor to be evaluated and receive routine care. Physical risks are those associated with drawing blood from a large vein in the arm, being given a small intravenous dose of a non-toxic zinc tracer, and of taking a small one tablet dose of a common muscle relaxant, chlorzoxazone.

To keep the risks very small the blood drawing will be done by a highly experienced professional who draws blood from adults almost daily. The total amount of blood taken during the study will be less than 400 mL (about 14 oz), a relatively small amount for an adult, and an amount that is substantially less than the amount given during blood donation. Rarely the process of drawing blood causes pain or bruising. Infection is very rare.

The administration of the zinc tracer to humans involves very little risk. Zinc is an essential nutrient. About 4 % of zinc in nature occurs in the form zinc that is used in this project as a tracer. The tracer is non-radioactive. It is handled in the body like all other zinc. Two milligrams of the tracer is dissolved in a very dilute salt solution and given intravenously. This amount is similar to the zinc requirement of an adult for one day. This amount is a common component of intravenous nutrition preparations that are used every day in hospitals. The tracer will not cause fever or infections.

Chlorzoxazone is a muscle relaxant medication that is used by doctors to treat painful muscles. The usual dose is 1500 mg to 3000 mg daily. The amount of chlorzoxazone administered in this project, 500 mg, will be 1/3 of the therapeutic dose. Infrequent side effects of the therapeutic dose include gastrointestinal distress, drowsiness, dizziness, lightheadedness, and gastrointestinal bleeding. The amount given to the subjects in this project are unlikely to cause these effects. Precautions will exclude subjects in the study who have known intolerance to chlorzoxazone or who are taking central nervous system depressants.

There are no obvious psychological or social risks associated with this project. Confidentiality will be maintained by keeping records in a locked file. Health records will be in the subjects hospital chart.

The data obtained from this study will be used for research purposes here at the University of Texas Medical Branch, Galveston, Texas, and with other collaborators at the United States Department of Agriculture (USDA), Grand Forks, North Dakota and at the Department of Social and Preventive Medicine - Kyoto University - Graduate School of Medicine, Kyoto, Japan. We will maintain total confidentiality by numbering subjects so that others cannot identify them.

BENEFITS TO THE SUBJECT

I understand that the direct benefits to me include a medical screening evaluation, and the possibility that any nutritional deficiencies I might have will be temporarily corrected.

REMOVAL FROM THE STUDY

I understand that I may be removed from this study at the Principle Investigators discretion.

ALTERNATIVE TREATMENT

N/A. There is no alternative procedure/treatment.

REIMBURSEMENT FOR EXPENSES

I understand that I will be reimbursed for lost time, travel, parking, meals, etc. in the amount of $25 when completing phase 1, $100 when completing phase 2, $125 when completing phase 3, and $175 when completing phase 4.

COSTS OF PARTICIPATION

I understand the study will involve my time and two nights stay in the CRC on four occasions. There will be some discomfort with placement of tubes in veins, and the muscle function testing but this is minimal.

UTMB STANDARD CLAUSES

1. I understand that informed consent is required of all persons in this project.

2. The principal and alternate procedures, including the experimental procedures in this project, have been identified and explained to me in language that I can understand.

3. The risks and discomforts from the procedures have been explained to me.

4. The expected benefits from the procedures have been explained to me.

[redacted] Initial Date

5. An offer has been made to answer any questions that I may have about these procedures. If I have any questions before, during or after the study, I may contact Dr. Harold H. Sandstead at (409) 772-4661.

6. I have been told that I may refuse to participate or stop my participation in this project at any time without prejudice and without jeopardizing my medical care at UTMB. All new findings during the course of this research which may influence my desire to continue or not to continue to participate in this study will be provided to me as such information becomes available.

7. I have been told that The University of Texas Medical Branch at Galveston, like virtually all other Universities in the United States, does not have a mechanism for compensation of the injured research subject. Therefore, I understand that I cannot look to any such mechanism to receive financial remuneration for any such injuries resulting from my participation in this project.

   I understand that I am authorized all necessary medical care for injury or illness which is the proximate result of my participation in this research. UTMB must provide such medical care when conducting research on private citizens. Other than medical care that may be provided (and any other remuneration specifically stated in this consent form), there is no other compensation available for my participation in this research study; however, I understand this is not a waiver or release of my legal rights.

8. If I have any questions regarding my rights as a patient participating in this study or research-related injury, I may contact Wayne R. Patterson in Research Administration Services at (409) 772-3482.

9. I have a right to privacy, and all information that is obtained in connection with this study and that can be identified with me will remain confidential as far as possible within state and federal law. However, information gained from this study that can be identified with me may be released to no one other than the investigators, research collaborators and my physician. The results of this study may be published in scientific journals without identifying me by name.

10. It is the policy of the U.S. Army Medical Research and Material Command that data sheets are to be completed on all volunteers participating in research for entry into this Command's Volunteer Registry Data Base. The information to be entered into this confidential data base includes you name, address, Social Security number, study name and dates. The intent of the data base is two-fold: first, to readily answer questions concerning an individual's participation in research sponsored by USAMRMC; and second, to ensure that the USAMRMC can exercise its obligation to ensure research volunteers are adequately warned (duty to warn) of risks and to provide new information as it becomes available. The information will be stored at USAMRMC for a minimum of 75 years.

11. Representatives of the U.S. Army Medical Research and Material Command are eligible to review research records as a part of their responsibility to protect human subjects in research.

I voluntarily agree to participate as a subject in the above named project. I understand that I will be given a copy of the consent form I have signed.

Initial Date

Date

Signature of Subject

Type/Print Name of Subject

Type/Print permanent address of Subject

Date

Signature of Witness

Type/Print Name of Witness

Type/Print permanent address of Witness

Using language that is understandable and appropriate I have discussed this project and the items listed above with the subject and/or his authorized representatives.

Date

Signature of Project Director
or his/her Representative

Zinc-Iron study: Principle Investigator: Sandstead, Harold H.

[redacted]

Initial Date

SIMPLIFIED PRETREATMENT METHOD FOR THE ANALYSIS OF PLASMA SAMPLES APPLICABLE TO ZINC KINETIC STUDIES

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ABSTRACT

Common biochemical indicators of zinc, such as plasma Zn are insensitive. Prasad et al (1963) utilized ^{65}Zn to show the rapid plasma Zn disappearance in Zn deficiency. Radiation exposure limits its application. Stable Zn isotopes are alternatives. Sample purification is usually required to obtain accurate results for mass spectrometric analysis, but also increases the chance of contamination. We compared two pretreatment methods (extraction vs nonextraction) for Zn isotope ratio (IR) measurement by inductively coupled plasma-mass spectrometry (ICP-MS). Plasma samples collected from ten human subjects 5 min to 24 h and four subjects 5 min to 9 days after 2 mg ^{67}Zn i.v. dose were used for comparison. The plasma (1.5 ml) was digested by hydrogen peroxide (Alcock, 1987) and dissolved in nitric acid. **"Extraction":** Zn in the digestate was extracted into CCl_4 as diethylammonium diethyldithiocarbamate chelate followed by back extraction of Zn in nitric acid. The solution was then heated overnight at 80°C to remove traces of CCl_4, and made up to 10 ml with high purity water after adding yttrium (Y) internal standard. **"Nonextraction":** Y and high purity water was directly added to the digestate. After subtraction of the baseline the plasma Zn IR was divided by the natural Zn IR to give the normalized IR (NIR). Logarithmically transformed NIR was calculated for regression analysis. NIRs $^{67/66}Zn$ and $^{67/68}Zn$ obtained from the extracted samples agreed well (r^2= 0.998). The NIRs obtained from $^{67/68}Zn$ and $^{67/68}Zn$ by both the methods agreed well compared to those from other ratios ($^{67/64}Zn$, r^2=0.838; $^{67/66}Zn$, r^2= 0.976; $^{67/68}Zn$, r^2=0.985; $^{67/70}Zn$, r^2=0.747). Considering the minimum possibility of isobaric interferences in plasma samples, $^{67/68}Zn$ obtained from nonextracted samples is sufficient for routine Zn kinetic analysis.

INTRODUCTION

For the assessment of Zn nutriture, determinations of plasma/serum Zn concentrations are used extensively in clinical practice, with normal plasma/serum Zn concentrations generally considered to be >7 umol/L. However, these concentrations are known to be influenced by many physiological factors (Goldenberg et al., 1995). There is a need for improved techniques to assess Zn status and monitor Zn metabolism since the Zn content of accessible tissues does not appear to provide a reliable index of Zn status and plasma or serum zinc concentrations vary with stress conditions unassociated with Zn deficiency. Isotopic techniques seem to provide an answer to this problem.

Radioisotopes of Zn have been used to develop complex mathematical models which describe Zn kinetics under various conditions in man and laboratory animals (Foster et al, 1979; Henkin et al., 1984; Wastney et al, 1986; Dunn and Cousins, 1989). Such models have been used to identify sites of regulation of Zn metabolism and calculate the size and turnover rate of body Zn pools. A simpler model describing Zn kinetics over a short time period (90 minutes) has been developed using ^{65}Zn in the rat (Lowe et al, 1991). Using this model it was shown that a rapidly exchanging pool of Zn is responsive to changes in dietary Zn intake, becoming significantly depleted in animals maintained on a Zn deficient diet. ^{65}Zn has a biological half-life of 500 days (Hawkins et al., 1976), and hence its applicability in the study of Zn metabolism in animals or humans is debatable.

A potential approach to the study of relationships between dietary Zn supply and body status is the measurement of plasma Zn kinetics following an intravenous injection (i.v.) of a stable Zn isotope. Stable isotopes offer a clear advantage over radioisotopes in that there are no ethical problems with administering it to the subjects. Recent studies in the literature have shown that stable Zn isotopes can be used to study Zn kinetics in humans, and such studies have been primarily used to assess rates of Zn absorption and gastrointestinal excretion (Turnlund et al., 1982, 1984, 1986; Istfan et al., 1983, Miller et al., 1994), although Jackson et al. (1984, 1988) used ^{67}Zn to examine Zn turnover rates in humans. Several instrumental techniques have been used in the literature for the determination of isotope ratios of Zn including neutron activation analysis (Janghorbani et al., 1980; Gokman et al., 1989; Wastney et al., 1986, 1991) and mass spectral methods such as thermal ionization (Fairweather-Tait et al., 1993), fast atom bombardment

(Peirce et al., 1987, Friel et al., 1992; Miller et al., 1994; Sian et al., 1996), and inductively coupled plasma (Serfass, 1986, Lowe et al., 1993, Friel et al., 1993)

Recently, our laboratory (Yokoi et al., 1994a,b) and a few other laboratories (Jackson et al., 1988; Lowe et al., 1991, 1993) have shown that stable isotopes of Zn can be successfully used to measure Zn turnover rates (TR) and exchangeable Zn pools (EZP) which are responsive to changes in Zn status. The availability of and ability to measure stable isotopes of Zn by mass spectrometry make this a viable technique for Zn metabolic studies. Studies from our laboratory indicate that inductively coupled plasma-mass spectrometry (ICP-MS) provides reliable detection of the isotopes of Zn (Yokoi et al., 1994a,b).

The stable isotope kinetics methodology for the determination of Zn status involves: (i) the use of intravenously administered Zn-67 stable isotope tracer, collection of blood at various time points, and (ii) determination of isotope ratios $^{67/64}Zn$, $^{67/66}Zn$, $^{67/68}Zn$, and $^{67/70}Zn$ at each time point using ICP-MS. The data are used to calculate Zn disappearance and turnover rates, and the exchangeable Zn pools after injection. The findings are related to other biochemical and neuropsychological functions in order to establish zinc status.

Extraction of Zn from samples is usually required to obtain accurate results for mass spectrometric analysis (Serfass et al., 1986; Miller et al., 1994; Yokoi et al., 1994 a,b). Unfortunately, the extraction also increases the chances of contamination since Zn is ubiquitous in the environment. The major matrix elements in human blood samples are sodium (Na, 3250 ppm), chloride (Cl^-, 3500 ppm), and sulfur (S, 1200 ppm) (Vanhoe et al., 1989). Theoretically there are no apparent major isobaric interferences for ^{66}Zn and ^{68}Zn in blood plasma, although $^{32}S^{16}O_2$ and $^{32}S_2$ overlap ^{64}Zn. Hence, we wanted to test this theory by conducting a detailed study of comparing the isotope ratios obtained from several sets of "extracted" and "nonextracted" plasma samples. Basically, this paper compares the four different isotope ratios obtained from Zn-extracted and Zn-nonextracted digested plasma samples in order to investigate whether the "nonextraction" procedure (with little or no contamination) is applicable for routine Zn isotope ratio analysis for kinetic studies in humans.

EXPERIMENTAL

Chemicals, Reagents and Supplies

The enriched stable isotope ^{67}Zn (as oxide, purity 93.11%) was purchased from Oak Ridge National Laboratory (Martin Marietta Energy Systems, Inc., Oak Ridge, TN, U.S.A.). Hydrogen peroxide (30%, ultrapure), nitric acid (ultrapure double-distilled from Vycor), ammonium hydroxide (high purity grade), and hydrochloric acid (ACS grade) were purchased from GFS Chemicals, OH, U.S.A.). Carbon tetrachloride (ACS grade) and 2,6-dinitrophenol, and Zn and yttrium reference standard solutions were purchased from Aldrich Chemical Co., Milwaukee, WI, U.S.A. Diethylammonium diethyldithiocarbamate was purchased from Tokyo Casei Co., Tokyo, Japan.

Deionized water was prepared using a Milli-Q System (Millipore Corp., Milford, MA, U.S.A.). Monovette syringes containing lithium heparin (10 U/mL blood) used for blood collections and polypropylene sterile tubes (29 x 114 mm size with red caps) free from Zn contamination used for plasma digestions were purchased from Sarstedt Inc., Newton, NC, U.S.A. Disposable Falcon polypropylene tubes (15 mL capacity) used for preparing the final ICP-MS digestate solutions and absolute ethanol used to dissolve the 2,6-dinitrophenol indicator were purchased from Fisher Scientific Co., Pittsburgh, PA, U.S.A. The carbon tetrachloride extraction of Zn from the digestates were carried out in borosilicate glass tubes (Kimax Inc., Toledo, OH, U.S.A.).

Human Subjects and Zinc Kinetics

Five fairly healthy men and one woman living in Galveston, Texas were the subjects for the 9-day observation. The other eleven women from the on-going Department of Defense study participated in 24-hour observation study. This study was approved by the Institutional Review Board of the University of Texas Medical Branch and written consents were obtained from all subjects.

The disappearance rate for ^{67}Zn from blood plasma, turnover rate, and the exchangeable Zn pool sizes were measured using the procedures well

established in our laboratory (Yokoi et al., 1994 a,b; Yokoi et al., 1997; Sadagopa Ramanujam et al., 1997; Egger et al., 1997).

Zinc kinetics were measured using ^{67}Zn (natural abundance 4.11%; enrichment, 93.11%) chloride which was prepared from ^{67}Zn oxide by dissolving 59.52 mg in a few drops of concentrated hydrochloric acid (ACS grade, GFS Chemicals, Columbus, OH), and heating it to dryness on a hot plate. The synthesized chloride was dissolved in saline (12 mL, corresponds to 0.5 mL = 2 mg of ^{67}Zn), aliquots of 0.5 mL sterilized by passing the solution through Millipore filter (0.2 uM pore size) into glass vials containing 10.0 mL saline. Several of these vials (one per 10 vials) were randomly selected and tested for sterility (University of Texas Medical Branch, Department of Clinical Microbiology and Immunology) and pyrogenicity (Scientific Associates Inc., St. Louis, MO).

Subjects were admitted to the Clinical Research Center before the test. The diet was limited in bioavailable zinc. At 07:00 A.M., after the subject had fasted at least 12 hours, short Teflon catheters connected to a 3-way stop cock were placed in each anticubital vein and kept open by 0.9% saline solution. After 30 min, a blood sample was taken to establish the baseline $^{67/68}Zn$ ratio.

Then the ^{67}Zn tracer - 2 mg in 10 mL saline further diluted to 30 mL in saline - was administered over 3 min (timed by stop watch). The line was flushed rapidly with saline for 30 seconds. Blood samples were then collected from the opposite arm starting 5 min after completion of the ^{67}Zn administration. Additional samples were collected at 5,15, 30, 40, 50, 60, 90 minutes, and 2, 6, 12 hours, and 1, 2, 3, 5, 7, and 9 days later. The 9-day samples were collected from 4 subjects out of 14. The 1-day samples from 10 out of 14. Blood samples were placed in an ice chest during the collection and promptly delivered to the laboratory for processing.

Digestion of Plasma and Extraction of Zinc

Sample digestion was based on the method of Alcock (1987). Duplicate aliquots of plasma were measured out in 50 mL polypropylene tubes, kept overnight at -70°C, transferred to a freeze-drier and lyophilized overnight, further dried for 8 hours at 80°C in an oven, and digested with 30% hydrogen peroxide (2 aliquots of 5 and 7 mL, high purity grade, GFS Chemical Co.) for 2

days at 85-90°C. The white ash was dissolved in 1.5 mL 1.2N Ultrapure nitric acid. Several (4 tubes/batch) hydrogen peroxide blanks run throughout the entire procedure were used to calculate ICP-MS IR blank subtractions.

"Extraction": The extraction of Zn was based on the method of Serfass et al (1986) suitably modified by Yokoi et al (1994). After digestion of the plasma, the ash was dissolved in 1.5 mL of 1.2 M nitric acid and the solution transferred to 20 mL acid-washed borosilicate tube using the Zn-free polyethylene transfer pipette followed by two washes with deionized water. One drop (40 uL) of 0.1% 2,6-dinitropehenol in 50% ethanol was added to the solution as a pH indicator. Dilute ammonium hydroxide was added in drops with shaking the tube to bring the pH to 2.5 (indicated by the color change to yellow). One mL of 0.25% diethylammonium diethyldithiocarbamate in carbon tetrachloride was added, the tube closed tight with Teflon-lined cap, and the contents shaken vigorously for 2 minutes. Each tube was allowed to stand until separation of the acid and carbon tetrachloride layers was complete.

The carbon tetrachloride layer containing chelated zinc was transferred to another glass tube carefully using the acid-washed glass pasteur pipette, the Zn-chelate decomposed with 1 mL of 1.2 M nitric acid, and the Zn back-extracted into the acid by vigorously shaking the tube. The back-extration of Zn was usually indicated by the transfer of yellow color into the acid layer followed by its disappearance. If such a transfer did not occur immediately, the solution was allowed to for an hour and shaken again to complete the decomposition and transfer steps. Then the top acid layer was transferred to another glass tube and the solution heated overnight at 80°C to remove traces of CCl_4, and made up to 10 ml with Milli-Q deionized water after adding yttrium internal standard (100 uL of 5 mg/L solution in 1% nitric acid).

"Nonextraction": After digestion of the plasma, the ash was dissolved in 1.5 mL of 1.2 M nitric acid and each solution transferred to a polypropylene falcon tube followed by two washes with Milli-Q deionized water. The Yttrium internal standard and Milli-Q water were directly added to the digestate and made up to 10 mL with water.

Inductively Coupled Plasma-Mass Spectrometry (ICP-MS)

A VG PlasmaQuad-1, upgraded to PlasmaQuad-2 plus status (VG Instruments, Winsford, England, U.K.) ICP-MS instrument was used for all isotope ratio measurements. Each solution was aspirated and nebulized (Meinhardt concentric type) into the argon plasma (8000-6000° K) via a peristaltic pump with a flow rate of approximately 1 mL/min. The yttrium internal standard was used to correct errors due to instrumental drifts during data acquisitions. Isotope ratio analyses were performed using "Peak-Jump Acquire" data acquisition mode of the software. The peak-jump acquisition mode gave better relative standard deviations (RSD <1%) compared to the scan acquisition mode (2-4%). The mass range scanned was 50-95 amu with 200 scan sweeps of 2048 channels, 160 microsec dwell time per channel, and 200 peak jump sweeps with 10240 microsec per peak jump sweep. These mass spectral acquisition parameters normally require about 9 mL of solution and 25 minutes acquisition time for ten replicate measurements of each sample. Instrument control, methods procedures and the data system, including calculations and statistics, were operated via a Compaq AT personal computer with version 3.2 of the V.G. PlasmaQuad software . All the Four Zn isotope ratios ($^{67/64}$Zn, $^{67/66}$Zn, $^{67/68}$Zn, $^{67/70}$Zn) were measured in each sample. The mass discrimination among Zn isotopes was corrected by the frequent measurements of Zn standard solutions (125, 250, and 500 ng/mL) during the sequence of IR analysis.

Calculations

Subtraction of the hydrogen peroxide mass spectral signal counts from each sample counts gave the blank-subtracted counts. Using the blank-subtracted signal counts, the four $^{67/64}$Zn, $^{67/66}$Zn, $^{67/68}$Zn, $^{67/70}$Zn IR values were recalculated for each sample. The value obtained after subtraction of the baseline (zero time) IR from each IR value was divided by the natural Zn IR value to obtain the normalized IR (NIR) value. A data set of 4 normalized isotope ratios (67/64, 67/66, 67/68 and 67/70) x 163 time points x 2 treatments (extraction and nonextraction)obtained from 14 subjects after iv dose of ^{67}Zn was subjected to statistical analysis. All statistical analyses were carried out using the SYSTAT5 (version 5.2.1) Macintosh software (SYSTAT Inc., Evanston, IL).

From the semilogarithmic plot of NIRs versus plasma collection time (0 to 24 hours), the following kinetic parameters were calculated as follows: (a) the disappearance rate constant was calculated from the 30 min to 60 min slope, and (b) the zinc turnover rate by multiplying the initial slope with plasma zinc central compartment size obtained from the 'truncated model'. The 24-hour spot plasma pool size was calculated from the equation :

Spot pool size = Dose of iv tracer/NIR x Natural abundance of ^{67}Zn (Yokoi et al, 1997).

RESULTS AND DISCUSSION

The inductively coupled plasma-mass spectrometry has become a powerful alternative for the determination of isotope ratio measurements along with other well established techniques such as neutron activation analysis and thermal ionization and fast atom bombardment mass spectrometry . However, when biological material is analyzed by ICP-MS, potential interferences from polyatomic ions must be considered. These interfering polyatomic ions originate mainly from argon, nitrogen, and/or oxygen in combination with sodium, sulfur, chlorine, and calcium, which are present at approximate concentration ranges of 3130-3370, 1120-1270, 2940-4120, and 92-109 mg/L in human serum, respectively (Vanhoe et al., 1989). Zinc has five isotopes: 64, 66, 67, 68, and 70. The most abundant isotope, ^{64}Zn(48.9%), is interfered to a larger extent by polyatomic ions containing sulfur, oxygen, and calcium. Table 1 summarizes the possible polyatomic ions which might interfere with Zn nuclides in serum solution. By using simulated solutions containing similar concentrations of Na, Cl^-, S, and Ca as in serum, the approximate ranges of total polyatomic interferences from S, O, and Ca in 5-fold diluted samples have been estimated to be 855-891 and 55-68 ug/L for ^{64}Zn and ^{66}Zn, respectively (Vanhoe et al., 1989).

Table 2 lists the range of NIRs for the four isotope ratios chosen. When Zn in samples are extracted, Zn isotopes 64, 66 and 68 can be used as a denominator isotope to calculate normalized isotope ratio. However, the low abundance of ^{70}Zn does not allow an accurate measurement of normalized isotope ratio even after the extraction of Zn. As expected, all the NIR values were found to be the lowest after 9 days of i.v.

administration of ^{67}Zn and highest at 5 minutes after injection. Negative values are irrational because all NIRs were obtained only after the administration of ^{67}Zn. The frequency of negative values for NIR was found to be very low; 2 out of 163 values (1.2%) for NIR-A $^{67/70}Zn$ and 7 out of 163 values (4.3%) for NIR-B $^{67/70}Zn$. Negative values were not observed for NIRs obtained from $^{67/66}Zn$ and $^{67/68}Zn$.

Table 3 shows the correlations (r^2) between different NIRs obtained from extracted samples using simple linear regression and double logarithmic (power function fitting) plots. As expected, the correlations are very high for isotopes 64, 66, 67, and 68 due to the removal of the interfering polyatomic background ions during the extraction of Zn. Figure 1 shows the correlation (double logarithmic plot, $r^2 = 0.998$) of NIRs of $^{67/68}Zn$ versus $^{67/66}Zn$ for the extracted samples.

Table 4 compares the normalized isotope ratios obtained from the extracted (NIR_A) and nonextracted (NIR_B) samples using simple linear regression and double logarithmic (power function fitting) plots. NIR_B calculated from 67/68 and 67/66 agrees very well with NIR_A. The extent of agreement of A and B batches for 67/68 is followed by 67/66. In contrast, NIR_B calculated from 67/64 and 67/70 poorly agreed with NIR_A.

Figures 2 and 3 correlate the NIRs of "extracted" versus "nonextracted" samples for $^{67/68}Zn$ ($r^2 = 0.985$) and $^{67/66}Zn$ ($r^2 = 0.976$), respectively. The regression analyses values (r^2, the slope "a", and the intercept "b") for NIR correlations from both the "extraction" and "nonextraction" methods show high correlations for $^{67/68}Zn$ and $^{67/66}Zn$. Such high correlations for "nonextracted" samples can be routinely achieved by: (a) keeping the resolution of the mass spectrometer between 0.8 and 0.9 amu, (b) cleaning the skimmer/sampling cones, torch, and the nebulizer prior to analysis of each batch of samples, and (c) passing nitric acid (1%) between the samples until the ^{89}Y (internal standard) signal reaches below 2000 counts . The resolution of the mass spectrometer is crucial to reduce the unexpected backgrounds.

However, the correlations are poor for $^{67/64}Zn$ ($r^2 = 0.838$) and $^{67/70}Zn$ ($r^2 = 0.747$) due to sulfur and oxygen polyatomic ion (mostly $^{32}S^{16}O_2$ and $^{32}S_2$) backgrounds at ^{64}Zn mass and very low sensitivity for ^{70}Zn ,

respectively. Considering the possibility of isobaric interferences generated during the ionization processes of the digested plasma samples inside the inductively coupled plasma of the ICP-MS coupled with this detailed investigation indicate that $^{67/68}Zn$ NIRs (with the least possibility of background ions) obtained from "nonextracted" samples are sufficient for routine Zn kinetic analysis.

To obtain accurate values for all the four Zn IRs, , it is essential to determine isotope ratio measurements using one set of "Extracted" and another set of "Nonextracted" plasma samples, since the extraction procedure involves many steps and some steps are susceptible to contamination. Our laboratory experience indicates that such a rigid duplicate analyses will establish unequivocally the NIR values and thus disappearance rates, exchangeable zinc pool sizes, and zinc turnover rates.

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Table 1. Possible Polyatomic Ions Interfering with the Nuclides of Zinc

Nuclide (% isotopic abundance)	Polyatomic Ions (major amounts)	Polyatomic Ions (minor amounts)
^{64}Zn (48.9)	$^{32}S^{16}O_2$, $^{32}S_2$	$^{46}Ca^{18}O$, $^{48}Ca^{16}O$
	$^{23}Na_2{}^{18}O$	$^{46}Ca^{17}O^{1}H$
^{66}Zn (27.8)	$^{32}S^{16}O^{18}O$	$^{48}Ca^{18}O$
	$^{32}S^{34}S$	$^{32}S^{17}O_2$, $^{33}S^{16}O^{17}O$,
		$^{34}S^{16}O_2$, $^{33}S_2$

Table 2. The range of Normalized Isotope Ratios (NIRs)

	"Extracted" Samples			
	67/64	**67/66**	**67/68**	**67/70**
Minimum	0.04	0.06	0.06	-0.43
Median	0.78	0.75	0.72	0.68
Maximum	14.33	13.67	12.91	12.89

	"Nonextracted" Samples			
	67/64	**67/66**	**67/68**	**67/70**
Minimum	-0.160	0.05	0.06	0.05
Median	0.53	0.71	0.75	0.69
Maximum	12.42	13.23	12.69	12.15

Minimum NIR was found 9 days after i.v. dose of ^{67}Zn.
Maximum NIR was found 5 minutes after i.v. ^{67}Zn administration.
Negative values are irrational because all NIRs were obtained after administration of ^{67}Zn.

Table 3. Correlations (r^2) between different NIRs obtained from extracted samples using simple linear regression and double logarithmic (power function fitting) plots

	Simple Linear Regression Plot		
	67/66	**67/68**	**67/70**
67/64	0.996	0.994	0.887
67/66		0.999	0.889
67/68	-		0.802
	Double Logarithmic Plot		
	67/66	**67/68**	**67/70***
67/64	0.996	0.991	0.786
67/66		0.998	0.794
67/68			0.893

*Negative values were removed for calculation.

Table 4. Comparison of normalized Zn isotope ratios (NIRs) obtained from extracted (A batch, NIR_A) and nonextracted (B batch, NIR_B) samples using simple linear regression and power function fitting (double logarithmic) plots

	Simple Linear Plot		
NIR	**r 2**	**a**	**b**
67/64	0.838	0.059	0.175
67/66	0.983	0.985	0.040
67/68	0.985	0.964	0.035
67/70	0.747	0.907	0.132
	Double Logarithmic Plot		
NIR	**r 2**	**a**	**b**
67/64*	0.838	1.237	0.773
67/66	0.976	1.023	0.958
67/68	0.985	0.987	1.001
67/70*	0.747	0.966	0.903

Regression equation for the simple linear equation is: NIR_A = a NIR_B + b, where 'a' and 'b' are the slope and the intercept, respectively. For perfect correlations, 'a' should be equal to 1 and and 'b' should be zero.

Regression equation for the double logarithmic plot is: NIR_A = a NIR_B power b. If both the values completely agree, then 'a' and 'b' should each be equal to1.

*Negative values are removed because they do not allow fitting.

Figure 1.

Normalized Isotope Ratios of 67/66 Zn and 67/68 Zn for Extracted Plasma Samples

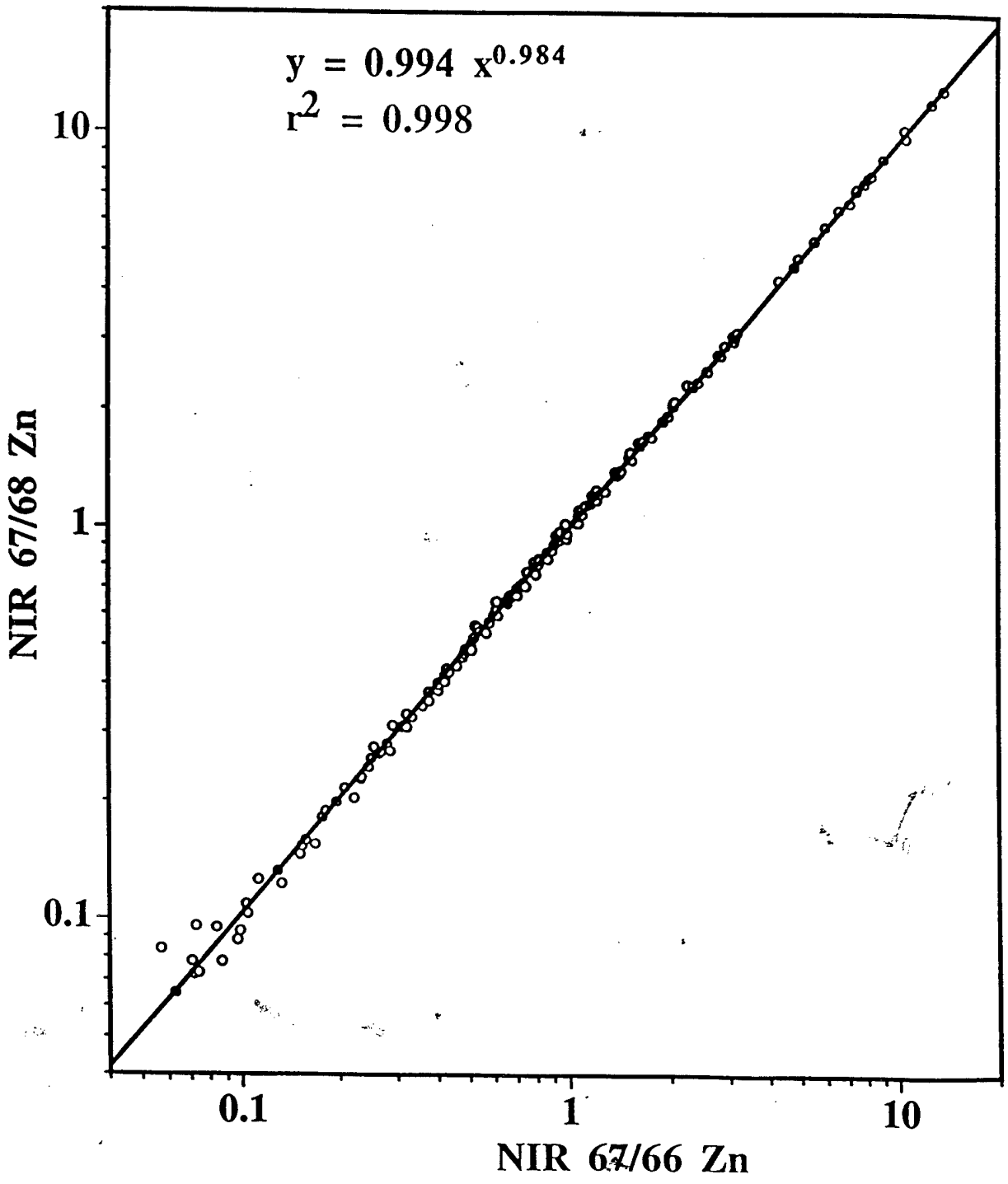


Figure 2.

Normalized Isotope Ratios of 67/68 Zn for Extracted and Nonextracted Plasma Samples

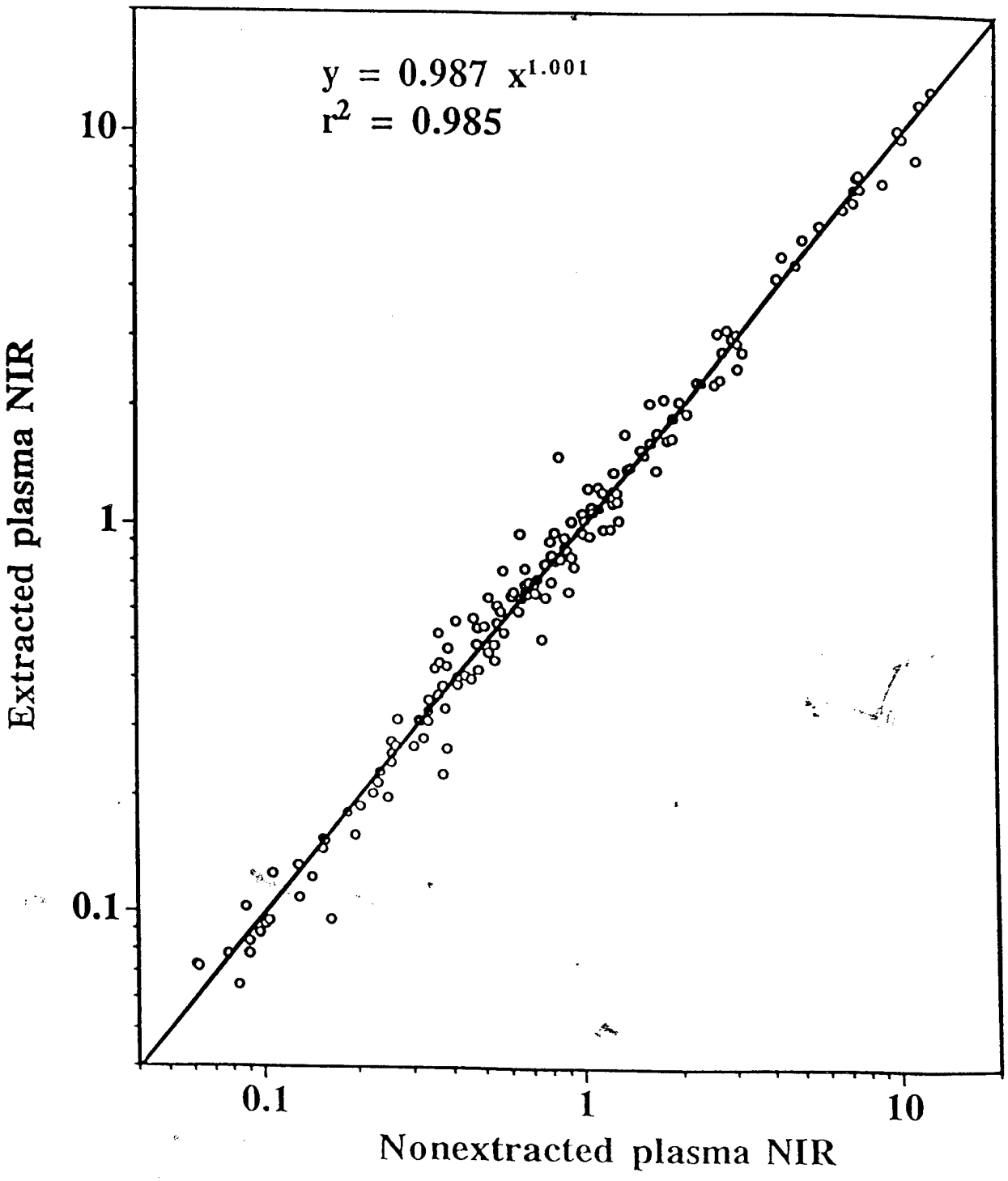
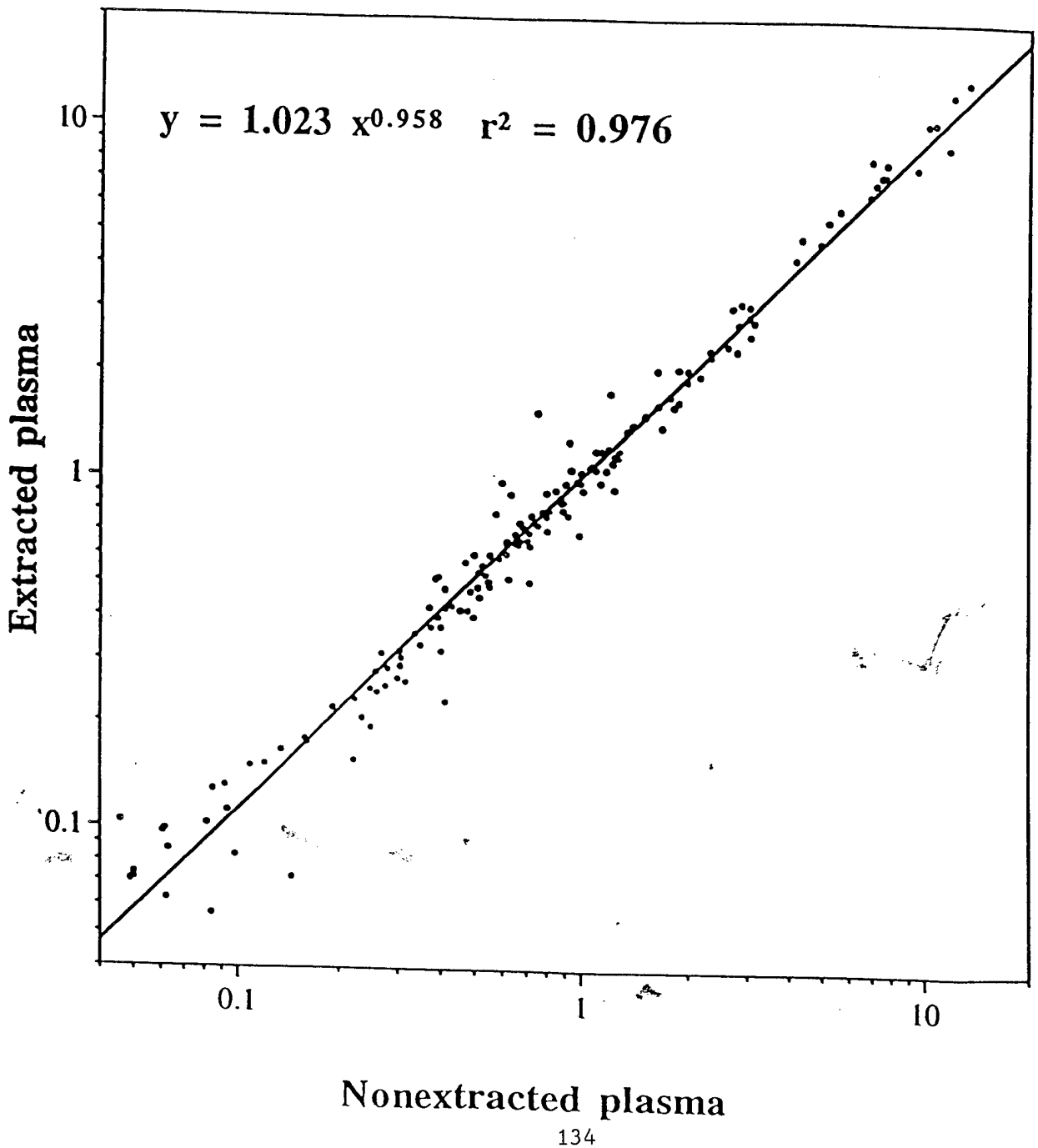


Figure 3.

Normalized Isotope Ratios of 67/66 Zn for Extracted and Nonextracted Plasma Samples



Comparison of Tri-Exponential and Truncated Models in Plasma Zinc (Zn) Kinetics.

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Abstract

Twenty-four-hour zinc (Zn) kinetics concordant with the nine-day kinetics was developed to show the validity of the 24-h spot plasma Zn pool as a practical indicator of so-called rapidly exchangeable Zn pool size (EZP). We compared kinetic parameters after iv dose of ^{67}Zn derived from 0 - 9 days (9-d model) and from 0 - 24 hours (24-h model) plasma collections in six subjects (5 men and 1 woman, age 24 - 64 y, BMI 23.2 - 30.4). Plasma Zn isotope ratios (IR) were measured by inductively coupled plasma - mass spectrometry. After baseline subtraction the plasma Zn IR was divided by the natural Zn IR to give the normalized IR (NIR). The tri-exponential function explained ($R^2 = 0.99$) NIR from 0 - 9 days = $K_1 \exp(-g_1 t) + K_2 \exp(-g_2 t) + K_3 \exp(-g_3 t)$, where t is time in days. Since the change of the third term during 24 hours was about 10 %, NIR from 0 - 24 hours was fitted ($R^2 =$ 0.98 - 0.99) by the above function when g_3 was constrained as 0. The estimated coefficients from the 9-d and 24-h kinetic models were similar except for g_2. The sum of three pools as a norm of EZP was calculated from the 0 - 9 day data using the three-pool models (mammillary and catenary; [Ramakrishnan, 1984 #811]) with a single outlet, which account for the loss of tracer from the system and the quasi-equilibrium between pools. We have shown the mathematical equivalency of the sum of three pools between the mammillary and catenary models, indicating that EZP is invariant by other models. The one-day spot plasma Zn pool was highly correlated (r^2=0.957) with the sum of three pools, suggesting that the one-day spot plasma Zn pool is a practical indicator of EZP. Turnover rate is practically estimable from the initial two pints (5 and 15 minutes) after iv dose of ^{67}Zn.

Introduction

Zinc is essential for many biochemical functions including protein synthesis and nucleic acid metabolism. It serves as a catalytic component of over 200 enzymes and as a structural component of various proteins, hormones, and nucleotides [Li, 1980 #85]. Sandstead et al reported behavior abnormalities in zinc deficient animals [Wallwork, 1993 #826] and the impaired cognition in humans with low zinc intake. Biochemical indices for evaluating Zn status are imperfect and the specificity of physiological indices is unknown. Zn kinetic parameters have been measured using radioactive Zn. Prasad et al [Prasad, 1963 #10] used ^{65}Zn to show rapid disappearance of plasma Zn in growth stunted adolescents. Using ^{69m}Zn and ^{65}Zn Aamodt et al [Aamodt, 1982 #704; Aamodt, 1979 #814] observed the change of Zn kinetics after oral loading of 100 mg Zn. Foster et al [Foster, 1984 #815] and Wastney et al [Wastney, 1986 #42] developed an integrated Zn kinetic model using ^{65}Zn and ^{69m}Zn.

Stable Zn isotopes are alternatives. Wastney et al [Wastney, 1991 #816] compared the results obtained from ^{65}Zn and ^{70}Zn using neutron activation analysis. Miller et al [Miller, 1994 #783] used stable Zn isotopes and FABMS [Peirce, 1987 #817] for measuring Zn pools. Using quadrapole inductively coupled plasma - mass spectrometry (ICP-MS), Lowe et al [Lowe, 1993 #62] analyzed the 120 min kinetics with ^{70}Zn and Yokoi et al [Yokoi, 1992 #486; Yokoi, 1994 #726; Yokoi, 1994 #481] evaluated Zn disappearance from 30 to 60 min after iv dose of ^{67}Zn. Fairweather-Tait et al [Fairweather-Tait, 1993 #818] measured Zn pools using ^{70}Zn and thermal ionization mass spectrometry (TIMS). Scott and Turnlund [Scott, 1994 #819] adapted Wastney's approach [Wastney, 1986 #42] to ^{67}Zn and ^{70}Zn tracer using TIMS.

There are two mathematical approaches. 1. The deconvolution method [Foster, 1984 #815; Wastney, 1986 #42] which treats remaining Zn tracer in plasma as a forcing function in the convolution integral [Berman, 1978 #820]. 2. The conventional compartment method based on coefficients in the polyexponential function fitted to the remaining tracer in plasma [Shipley, 1972 #768]. The kinetic parameters including the number of exponential terms depend on the observation intervals. We therefore investigated a short-term kinetic model concordant with the long-term kinetic model using ^{67}Zn and Quadrapole ICP-MS.

The rapidly exchangeable zinc pool (EZP) is believed to represent the metabolically active form of zinc and to relate with the physiological function of zinc [Wastney, 1986 #42; Miller, 1994 #783]. However the definition of EZP is not clear. We report the mathematical equivalency of the sum of three pools between the mammillary and catenary models [Ramakrishnan, 1984 #811], indicating that the sum of three pools is a well-defined invariant quantity of EZP.

Subjects and methods

Human subjects

Apparently healthy 5 men and 1 woman living in Texas were the subjects for the 9-day observation. The other 11 women from our on-going main project supported by Department of Defense participated in 24-hour observation study. The characteristics of the subjects were shown in Table 1. This study was approved by the Institutional Review Board of the University Texas Medical Branch and the written consents were obtained from all the subjects.

The ^{67}Zn oxide (59.52 mg) was dissolved in a few drops of concentrated hydrochloric acid and heated on a hot plate at 80°C to dryness. The obtained ^{67}Zn chloride was dissolved in 12 mL saline; 0.5 mL aliquots was sterilized by passage through Millipore filters (0.2 μm pore size) and added to 9.5 mL sterile saline in glass or plastic vials. Each vial contains 2 mg ^{67}Zn. These solutions were tested for sterility (UTMB Department of Clinical Microbiology and Immunology) and pyrogenicity (Scientific Associates Inc., St. Louis, Missouri).

After a 10 hour overnight fast short Teflon catheters were placed in both antecubital veins of the subjects. The catheters were attached to a slow drip of normal saline by a three-way-stop-cock. A baseline blood sample was taken for the $^{67/68}Zn$ ratio. Then 2 mg of sterile, pyrogen free, ^{67}Zn in saline was administered over three minutes through the stop-cock. This was followed by the rapid administration of saline from the drip for 3 minutes. Blood samples were taken from the other catheter at 5, 15, 30, 40, 50, 60 and 90 minutes, 2, 6, 12 and 24 hours, and (2), 3, 5, 7 and 9 days after the administration of ^{67}Zn. Amounts of blood taken at each time point were at least 10 mL. Before each blood collection about 2 ml blood was taken in a plastic syringe to wash the catheter. Blood samples were taken in a Monovette syringe containing lithium heparin (10 U/mL blood) obtained from Sarstedt. A 24 hour urine was collected for $^{67/68}Zn$ ratio. After emptying the bladder in the morning 1 hour spot urine samples were collected 2, 3, 4, 5, 6, 7, 8 and 9 days after the iv dose of ^{67}Zn. Blood samples were placed in an ice chest during the collection and promptly delivered to the laboratory for processing.

Laboratory wares and Reagents

The enriched ^{67}Zn (as oxide, purity 93%) was purchased from Oak Ridge National Laboratory, Martin Marietta Energy Systems, Inc., Oak Ridge, TN. Hydrogen peroxide (30%, ultrapure), nitric acid (ultrapure double distilled from Vycor), ammonium hydroxide, and hydrochloric acid (ACS grade) were purchased from GFS Chemicals, Ohio. Absolute ethanol was obtained from Fisher Scientific Co, Pittsburgh, PA (Please check might be Fisher Chemicals). Carbon tetrachloride

(ACS grade) and 2, 6 - dinitrophenol was purchased from Aldrich. Zn and yttrium reference standard solutions were purchased from Aldrich (?, please check). Diethylammonium diethyldithiocarbamate was obtained from Tokyo Kasei, Co., Tokyo, Japan. Deionized water for dilution of the samples was prepared using a Milli-Q system (Millipore Corp, Milford, MA, USA). Argon gas (99.9% high purity grade) was provided to the ICP-MS from a liquefied argon cylinder (Tri-Gas Industrial Gases, Freeport, TX, USA) capable of delivering at least 20 liter/min at a pressure of 80 psi. The carbon tetrachloride extraction of Zn from the digestate was carried out in borosilicate glass tubes (Kimax, Owens-Illinois Inc., Toledo, Ohio). Disposable Falcon polypropylene tubes (15 mL capacity) used for making multiple dilutions of the digestates were purchased from Fisher Scientific Co, Pittsburgh, PA, USA. Polypropylene sterile tubes (29 x 114 mm size with red caps) free from Zn contamination used for plasma digestion were purchased from Sarstedt Inc. of Newton, NC.

Chemical analysis

Sample digestion was based on the method of Alcock [Alcock, 1987 #27]. Duplicate 1.5 mL plasma samples were placed in large polypropylene tubes and lyophilized for 30 hours, followed by dry heating at 80-85 °C over-night in an oven. The third day, 5 mL hydrogen peroxide (30%) was added at room temperature, vortexed slowly to stop frothing and kept overnight at room temperature. The samples were then digested at 90-95 °C. Every two hours an additional 2 mL of hydrogen peroxide was added until a white ash is obtained. It took about 10 mL of hydrogen peroxide for complete digestion.

The extraction of Zn was based on the method of Serfass et al [Serfass, 1986 #821] with suitable modifications by Yokoi et al [Yokoi, 1994 #726]. The ash was dissolved in 1.5 mL of 1.2 M nitric acid. The dissolved solution was transferred in 20 mL acid-cleaned borosilicate tubes with Teflon-lined-caps using the Zn-free polyethylene transfer pipette followed by two washes with deionized water. One drop of 0.1% 2,6-dinitrophenol in 50% ethanol was added to the solution as a pH indicator. Dilute ammonium hydroxide was added to bring the pH to 2.5 (indicated by the color change to yellow). One mL of 0.25 % diethylammonium diethyldithiocarbamate in carbon tetrachloride was added and the contents mixed by vigorous shake for 2 minutes. The tubes were allowed to stand until separation of the acid and the carbon tetrachloride layers was complete. The carbon tetrachloride layer containing chelated zinc was removed and placed in a second tube. The carbon tetrachloride layer was extracted again with 1 mL of 1.2 M nitric acid by vigorous shaking to move the Zn into the acid layer. The acid layer was transferred to a polypropylene tube very carefully, 100 µL of 5 µg/mL of the yttrium standard solution (internal standard) was added, and the volume made up to 10 mL for ICP-MS isotope ratio (IR) analyses.

A VG PlasmaQuad-1, upgraded to PlasmaQuad-2 plus status (VG Instruments, Winsford, England, UK) ICP-MS instrument was used for all analyses. The solutions were aspirated and nebulized (Meinhardt concentric type) into the argon plasma (6000-8000 K) by a peristaltic pump with a flow rate of approximately 1 mL/min. The internal standard of yttrium was added to correct errors due to instrumental drifts during data acquisitions. The mass discrimination among Zn isotopes was corrected by the frequent measurements of a Zn standard solution (250 and 125 ppb) during the sequence of IR analysis. The peak-jump mode was used for IR acquisitions since it gave better relative standard deviations (RSD = < 1%) compared to the scan-acquisition mode. We used IR parameters that are similar to those of Yokoi et al [Yokoi, 1994 #726], which include: 160 μsec dwell time, 200 scan-sweeps and 100 peak-jump-sweeps with 10 consecutive measurements. The Zn isotope ratio $^{67}Zn/^{68}Zn$ was measured on each sample.

Calculation of the normalized isotope ratio

After baseline subtraction the plasma Zn IR was divided by the natural Zn IR to give the normalized IR (NIR). The spot pool size was calculated as follows:

Spot pool size = Dose of *iv* tracer / NIR / Natural abundance of ^{67}Zn

Mathematical analysis

Mathematical analysis of the data involved three phases. The initial phase was the development of the mathematical model to explain the disappearance of ^{67}Zn from plasma following a single intravenous administration during the restricted observation period. In short, this phase determined the number of exponential terms for the shorter observation period concordant with the longer observation period. In addition, this phase estimated the stability of the nonlinear regression using the Monte Carlo simulation.

The second phase of the mathematical analysis involved the determination of invariant kinetic parameters against various connections of pools, i.e., mammillary and catenary models.

The third phase of the mathematical analysis involved the application of the model to the analysis of data obtained from the human subjects.

All modeling was done on the Macintosh Powerbook 165C (Apple) using the SYSTAT5 for Macintosh, version 5.2.1 software (SYSTAT, Inc., Evanston, IL). A logarithmic transformation of the normalized isotope ratio was used to stabilize the random variation at fitting a polyexponential function to the disappearance data [Brown, 1993 #822].

RESULTS

The first phase of the mathematical analysis

Formulation

The disappearance of Zn tracer from plasma is considered to be explained by the four-exponential function [Prasad, 1963 #10; Miller, 1994 #783] as follows:

$$\textit{Tracer in plasma} = K_1 e^{-g_1 t} + K_2 e^{-g_2 t} + K_3 e^{-g_3 t} + K_4 e^{-g_4 t}$$

$$\textit{where} \quad g_1 > g_2 > g_3 > g_4$$

At the case of $g_1 >> g_2 >> g_3 >> g_4$ as generally found in the zinc kinetics, the truncated form of the polyexponential function (the bi- or tri-exponential function with a constant term) can be substituted for the complete form when the observation period is shorter than the half-life of the third terms. From the point of the view of the curve fitting, the truncated form should be used in stead of the complete form to avoid the hyper-parameterization and the shortage of the degree of freedom. In the extreme case, hyper-parameterization often causes inconvergence found in the nonlinear regression.

According to the Miller et al's [Miller, 1994 #783] model based on Wastney et al's report [Wastney, 1986 #42], g_1, g_2, g_3 and g_4 are 137.6, 3.564, 0.1106 and 0.00232, respectively. The corresponding half-lives are 7.25 min, 4.67 h, 6.26 d and 298.7 d. We therefore propose the truncated polyexponential model as follows:

For within 24 h, $\textit{Tracer in plasma} = K_1 e^{-g_1 t} + K_2 e^{-g_2 t} + K_3$

For within 28 d, $\textit{Tracer in plasma} = K_1 e^{-g_1 t} + K_2 e^{-g_2 t} + K_3 e^{-g_3 t} + K_4$

Table 1 shows the comparison of the values in Miller et al's model and the estimated parameters from the 5 min - 24 h values using the bi-exponential function with a constant term. The estimates well agreed with the model values, although the consideration is required that the above estimation is based on the calculated values from the model without the analytical noise.

Table 2 shows the result of the Monte Calro simulation. The simulation suggests that 1-2% CV in the measurement of the remaining tracer is acceptable to estimate the kinetic parameters if the Zn tracer disappears depending on the four-exponential function like Miller et al's model.

Miller et al [Miller, 1994 #783] developed a four-pool based on Wastney et al's study [Wastney, 1986 #42]. We also analyzed the mean remaining tracer in normal subjects' plasma after intravenous administration of ^{65}Zn reported by Wastney et al [Wastney, 1986 #42]. For the data from 0 to 28 days and 0 to 2 days, the truncated polyexponential functions are applied to fit the curve. Because their article does not include the data one day after administration of tracer, the two-day data were utilized. The value from 0 to 290 days was analyzed with the complete quadri-exponential function as a standard. The proportion parameters K_4 (for the term with 158 d of half-life) and K_3 (6.66 d) were respectively predicted from the 28 day and 2 day observation, which were

shorter than their corresponding half-lives.

The following part might not be essential because Prasad's data does not include time points shorter than 30 min.

Prasad et al [Prasad, 1963 #10] reported the average value of the remaining tracer in plasma for the normal and dwarf subjects from 30 min-14 d after administration of the tracer. They did not report the value for the time less than 30 min. Therefore, we applied the tri-exponential function without a constant term as a standard to the 14-day observation. For a shorter observation than 14 days, we fitted the bi-exponential function with a constant term (the truncated model) and the bi-exponential function without a constant, as was often seen in the literature [Lowe, 1991 #705; Lowe, 1993 #62].

When a constant term is included, the parameters in the complete tri-exponential model were estimated from the shorter observation. Otherwise, the large difference from the complete model occurred as was seen g_2. When the observed period was below 10 h, the estimation of g_2 got worse. Therefore, 24 h observation is considered necessary to obtain the accurate estimation of the second term. The estimated parameter K_3 from the complete model (0.5 h-14 d) and truncated model (0.5 h-24 h) was 0.1294±0.0086 and 0.1198±0.0114 (estimate±ASE), respectively. The both values were similar.

Therefore we applied the complete tri-exponential function for 9-day data and the bi-exponential function with a constant term for 24-hour data.

The second phase of the mathematical analysis

Calculation of the kinetic parameters in the mammillary model

Based on Landaw et al [Landaw, 1984 #808], the kinetic parameters in the mammillary model were calculated. Accepting the single outlet assumption as proposed by Miller et al [Miller, 1994 #783], all parameters in the mammillary model are uniquely determined (See Figure 1; **Appendix 1**). Otherwise, only fluxes between pools are uniquely determined [Landaw, 1984 #808].

Definition of EZP

Jackson et al [Jackson, 1988 #823] and Miller et al [Miller, 1994 #783] defined rapidly exchanging pools of zinc or rapidly exchangeable Zn pools (EZP) as a lump of the pools of Zn that exchange completely with plasma within 2 days. Because the system is open to the outside, the system does not reach the true isotopic equilibrium but can be in isotopic quasi-equilibrium for certain time interval (**Appendix 2**). The extent of the isotopic equilibrium (or tracer/tracee equilibrium in a broad sense), i. e., the extent of the mixing is evaluated using time vs the ratio of the isotopic enrichments between two pools. The ratio of isotopic enrichments in pool b to pool a (IER_b/IER_a) takes maximum value of 1.426 at t = 0.066 day and the 95 % maximum at t = 0.200 day. The ratio of isotopic enrichments in pool c to a (IER_c/IER_a) takes maximum value of 1.135 at t = 2.752 day and the 95 % maximum at t = 1.130 day. The ratio of isotopic enrichments in the lump of pools a, b and c to pool a (IER_{a+b+c}/IER_a) takes maximum value of 1.113 at t = 2.718 day and the 95 % maximum at t = 1.040 day. It is reasonable to conclude that pools b and c are 'completely' exchanged with pool a (plasma compartment) within 2 days. It is favored that at t = 1 day the ratio of isotopic enrichments in the lump of pool a, b and c to pool a (IER_{a+b+c}/IER_a) is 1.049 which is close to 1 (true complete exchange). The remaining tracer in the lump of pools a, b and c at t = 1 day is 81.2 %. This result supports the hypothesis that 24 hour spot plasma pool will give the good estimation of EZP.

Possible methods estimating EZP

Obtaining the "true" EZP requires continuously monitoring isotope ratio in plasma from the tracer administration until the time at infinity using the infinite number of terms in the polyexponential function. This method is ideal and impossible to conduct. However, the estimate that is obtained from the longer observation period and polyexponential function with more terms is closer to the true EZP [Green, 1990 #824; Ramberg, 1992 #825]. The frequent initial sampling and the longer observation period impose a severe limitation on the application of the tracer technique to human zinc metabolism. The first point was discussed by Miller et al, who stated the limitation of the application to the children. The second point limits the experimental design and does not allow the

change of dietary regimen or another condition during the observation period for the kinetic analysis, which premises the steady state. There is a need for the method estimating the EZP from the shorter observation period and the less sampling. The followings are the possible methods estimating EZP.

Method 1 (open three-pool): A sum of three pools in the open three-pool system (mammillary or catenary) calculated from the 9-day observation interval using tri-exponential function model, which is considered as norm because of the longest observation period and most samplings.

Method 2 (closed three-pool): A pool calculated from the reciprocal of K3 in the truncated exponential function model applied to the 24-hour observation period, which is equivalent to the sum of three pools in the closed three-pool system (mammillary or catenary).

Method 3 (constrained open-three pool): A sum of three pools in the open three-pool system (mammillary or catenary) with the parameter restriction that fixes g_3 at 0.115 (the empirical value) calculated from the 24-hour observation interval using tri-exponential function model.

Method 4 (last term of tri-exponential function): A pool calculated from the reciprocal of K3 of the third term in the tri-exponential function model.

Method 5 (simple extrapolation of Miller et al [Miller, 1994 #783]): A pool calculated from the reciprocal of the intercept obtained from the 3 to 9 day extrapolation to the time of the tracer administration (t = 0) using the simple exponential function.

Method 6 (24-hour spot plasma pool): A pool calculated from the reciprocal of the normalized isotope ratio in the spot plasma 24 hours after the intravenous administration of tracer.

Considering the pool estimated from the open three-pool method (method 1), other methods give the approximation of EZP. Restricted open-three pool method (method 3) is essentially a correction of the closed-three pool method (method 2) using the empirical average value of g_3. Restricted open three-pool method is tentative because g_3 might vary more than expected when the subject is in an abnormal condition. The first and the second terms are negligible from 3 days after the administration of the tracer, because g_1 and g_2 are larger than g_3. Last term method (method 4) utilizes K_3 of the third term calculated from the 5 minutes to 9 days data. Simple extrapolation method (method 5) of Miller et al [Miller, 1994 #783] approximates K_3 of the third term method from the 3 to 9 day data. The one-day spot plasma pool method (method 6) is the simplest method that requires just two sampling, i.e., one day spot and baseline and approximates the closed three-pool method.

Conserved parameters by matrix transformation of three pool systems of mammillary and catenary models

Because there is no theoretical or experimental basis that justifies the mammillary model as a "true" model, the catenary model is also possible for three-pool system (Figure 1). Therefore, we

investigated the invariant kinetic parameters over different models based on Ramakrishnan's matrix transformation [Ramakrishnan, 1984 #811]. His basic idea was derived from Berman's indistinguishable model [Ramakrishnan, 1984 #811]. As was proven in the **Appendix 3**, the following kinetic parameters are invariant:

1. Pool size of the central compartment (plasma Zn pool)
2. Sum of the rate constant from the central compartment (initial slope)
3. Flux from the central compartment (plasma Zn turnover rate)
4. Sum of the pool sizes (so-called rapidly exchangeable Zn pool, EZP).

In the following section, we will limit the discussion within the invariant parameters.

The third phase of mathematical analysis: Application to the analysis of data from the human subjects

Figure 2 shows the illustration of the fitting the curve to the nine-day data using tri-exponential function model. Table 4 shows the coefficients and R-square obtained by the nonlinear regression for the 9-day data using the tri-exponential function. K_3 and g_3 are less changeable compared to other coefficients.

Table 5 shows the determined coefficients and R-square by the nonlinear regression for the 24-hour data using the truncated exponential function model (bi-exponential function with a constant term). Table 6 shows the percent deviation of the coefficients of the truncated exponential function model (24-hour data) from the tri-exponential function model (9-day data). Except for g_2, the determined coefficients from the different model (i.e., different observation period) were similar.

Table 7 shows the indicators describing quasi-equilibrium in the open mammillary system. The average values of the indicators that describe the quasi-equilibrium between central compartment and rapidly exchanging pools are similar to the indicators found in Miller's mammillary model, except for the time when IER_{1+2+3}/IER_1 takes maximum. The notation of the subscripts '1, 2 and 3' of 'IER' for the subjects correspond to 'a, b and c' for Miller's mammillary model that uses four compartments rather than three compartments.

Table 8 shows the comparison of rapidly exchanging Zn pool (EZP) determined by various methods. Considering EZP determined from Method 1 (open three-pool model), overestimation was obvious in the estimates of EZP from Method 2 (closed three-pool model), Method 4 (last term of tri-exponential function), Method 5 (3 - 9 day extrapolation) and Method 6 (one-day spot plasma pool). Method 2 that constrained g_3 as 0.115 in the open three-pool model corrected the overestimation found in Method 2 that constrained g_3 as 0 in the open three-pool model because the closed three-pool model is mathematically special case of the open three-pool model.

Table 9 shows the percent deviation of EZP estimates from Method 1. The mean percent deviation was about 20 % for Method 2, 4 and 5. Because Method 3 corrected the overestimation, its mean percent deviation was close to 0. For Method 5 (3 to 9 day extrapolation) overestimation was larger than other methods. The percent deviation for subject 1 by Method 5 was very larger (66 %) than was observed in another subject. It might be due to the selected interval for simple regression analysis. When the data 2 days later was included (2 - 9 day extrapolation), estimated EZP was 242 mg and the slope was 0.107 day^{-1}. The slope was 0.086 day^{-1} for 3 - 9 day extrapolation, which was too smaller than 0.1198 day^{-1} of g_3.

The correlation coefficient between EZP by Method 1 as a norm and other methods were as follows: 0.988 ($p = 0.0002$) for Method 1; 0.601 ($p = 0.21$) for Method 3; 0.773 ($p = 0.07$) for Method 4; 0.173 ($p = 0.78$) for Method 5; and 0.961 ($p = 0.002$) for Method 6. When subject 1's EZP estimate by 2 - 9 day extrapolation was employed in place of 3 - 9 day extrapolation, the correlation coefficient became 0.654 ($p = 0.23$).

Table 10 shows the plasma Zn turnover rate, i.e., the sum of flux from the central compartment. Since the turnover rate is determined by the initial slope and the intercept, the turnover rate determined from the three different models (open three-pool, closed three-pool and the constrained open three-pool models) agreed very well. The correlation coefficient between the TR estimated from the initial two points (5 and 15 minutes) and the TR obtained from the open three-pool (as a norm) was 0.985 ($p = 0.0003$). The percent deviation for the estimate by the initial two points was - 4.6 ± 2.0 % (Mean ± SD).

Discussion

There are some limitations and demands of the kinetic study of zinc as found in other nutrients. 1: Chemical analysis of stable isotopes limits the observable period of tracer. 2: Number of blood sampling and observation period are limited by the experimental design and the acceptable load to the subjects. 3: The 'true' model of zinc kinetics is not established. 4: The model derived from the shorter observation period should be concordant with the model built based on the longer observation period.

Even in plasma or serum, zinc distributes in several compartments biochemically or chemically defined [Harris, 1989 #87]. We can expect the presence of several compartments for tissue zinc rather than single compartment. These situations make us assume that plasma zinc is in a single compartment, as customly done by others [Foster, 1984 #815; Wastney, 1986 #42; Fairweather-Tait, 1993 #818; Miller, 1994 #783]. When the number of subcompartments of plasma zinc derived from the different chemical species and the chemical equilibrium among subcompartments are

established, the analytical method of the plasma zinc disappearance data must be revised in the future.

Based on Miller's model [Miller, 1994 #783] and the analysis of Wastney's data [Wastney, 1986 #42], we have chosen tri-exponential function to fit the disappearance curve of ^{67}Zn from plasma. As a possible three-pool model with all parameters uniquely determined, the mammillary and catenary models with a single outlet from the central compartment were considered. When the discussion is limited to central compartment (plasma Zn compartment), EZP and TR, the mathematical analysis revealed that the type of model does not affect the results of parameter estimates. Until we will get the 'true' model or the appropriate approximation for the multi-compartment system, invariant parameters will avoid the model-based biased comparison.

Monte Calro simulation revealed that the 2% random error is acceptable to estimate the coefficients in the tri-exponential model (Table 2). Therefore, the ICP-MS routine analyses that produce the measurement error less than 1 % is suitable for Zn kinetic study considering the sample throughputs.

The analysis of Wastney's data [Wastney, 1986 #42] and our data have shown the appropriate number of terms for various observation periods (Table 3 to 6) that allows the concordance of the shorter observation period to the longer observation period. Therefore, we further investigated the method for elucidating the practical indicators of the kinetic parameters determined from data based on many time points over 9 days.

In the isotopic quasi-equilibrium, the ratio of isotope enrichment in the peripheral compartment to the central compartment is not monotonous and takes maximum at the specific time (Appendix 2). Therefore we selected the time when the ratio takes maximum as a time of quasi-equilibrium, because it is uniquely determinable (Table 2-1 in Appendix 2; Table 7).

As is shown in Table 5, the open mammillary system derived from 9 day data of ^{67}Zn disappearance from plasma reached 95 % maximum of the ratio of isotope enrichment in pool 1+2+3 (EZP) to pool 1 (plasma Zn or central compartment) 0.6 to 1.5 day later with the average of 1.1 day. These results guarantee the method estimating EZP and TR developed by the analysis of Miller's mammillary model. We guess that 'natural break point' at 2 days after iv dose of tracer proposed by Miller et al [Miller, 1994 #783] may be a literal description of the quasi-equilibrium later than 1 day.

The overestimation from various methods compared to the sum of pools turning over within 48 hours (i.e., the sum of pools 1, 2 and 3 for the open three-pool model) is originated from the quasi-

equilibrium and the loss of tracer from the system (Appendix 2; Table 7), as was described with words by Miller et al [Miller, 1994 #783]. Fortunately the effect of the quasi-equilibrium is relatively small for the estimation of EZP using one-day spot plasma pool because the IER_{1+2+3} / IER_1 at t = 1 day was close to 1 for the subjects. By this reason, the one-day spot plasma pool is a practically good indicator although its basis is just single time point.

As a definition, the turnover rate is a product of the initial slope of the time vs semilogarithmic plot of the isotope enrichment and the plasma Zn pool size calculated from the extrapolated intercept to t = 0. The contribution of the later time points is considered smaller than the initial points. The comparison among TR estimated from various methods revealed that the initial two points are practically enough to estimate TR (Table 10).

In conclusion, EZP and TR derived from the open three-pool model using 9-day data are invariant to the models (mammillary or catenary) and can be utilized as a norm for comparison among individual Zn kinetic parameters. Using the closed three-pool model concordant parameters to the longer observation period are obtained. One-day spot plasma pool is a practical indicator of EZP. TR can be practically estimated from the initial two points (5 and 15 minutes) instead of one-day or nine-day observation period.

Table 1. Parameter estimates of Miller et al's illustration. The remaining tracer in plasma compartment from 5 min - 24 h was calculated from Miller et al's model [Miller, 1994 #783]. The parameters were estimated from the 5 min - 24 h values using the bi-exponential function with a constant term are as follows:

Parameter	Model value	Estimated parameters from the truncated exponential function	Asymptotic standard errors
K1	0.9545	0.9538	0.0020
g1	137.6	137.4	0.2
K2	0.03046	0.03207	0.00006
g2	3.564	3.322	0.0185
K3	0.01443	0.01326	0.00004

Where, g_3=0.1106, K_4=0.000628, g_4=0.00232.

These parameters are out of the estimable.

Tracer in plasma in Miller' s model = $K_1e^{-g_1t} + K_2e^{-g_2t} + K_3e^{-g_3t} + K_4e^{-g_4t}$

Tracer in plasma in the truncated exponential = $K_1e^{-g_1t} + K_2e^{-g_2t} + K_3$

Table 2. Mean and SD of the estimated parameters from the Monte Calro simulation with 100 trials using Miller et al's model. Estimation was based on the values from 5 min-24 h theoretical values given 1 or 2% noise (constant CV) using normal random numbers. SD is the standard deviation of the estimates. CV gives the relative standard error of the estimates.

	1% Random error given			2% Random error given		
Parameter	Mean	SD	CV	Mean	SD	CV
K_1	0.9523	0.0119	1.25%	0.9510	0.0237	2.49%
g_1	137.2	1.2	0.87%	137.1	2.5	1.82%
K_2	0.03201	0.00037	1.16%	0.03196	0.00074	2.32%
g_2	3.318	0.105	3.16%	3.314	0.210	6.34%
K_3	0.01326	0.00024	1.81%	0.01325	0.00049	3.70%

Table 3. Parameter estimates from various observation periods obtained from the analysis of Wastney et al's data [Wastney, 1986 #42]

Parameter	0 - 290 days	0 - 28 days	0 - 2 days
K_1	1.18	1.18	1.17
g_1	131	131	130
K_2	0.0433	0.0427	0.0451
g_2	4.50	4.66	4.19
K_3	0.0136	0.0143	0.0136
g_3	0.104	0.118	
K_4	0.00215	0.00228	
g_4	0.00439		

Values will be changed after the analysis of KY 2.

Table 4. Determined coefficients and R-square for the tri-exponential model

Subject	R_2	K_1	g_1	K_2	g_2	K_3	g_3
1 DGA	0.996	8.796	101.4	0.5753	3.813	0.1912	0.1198
2 HSE	0.998	8.118	120.2	0.3659	5.612	0.1913	0.1057
3 JCB	0.996	13.82	160.5	0.4049	2.825	0.2122	0.1029
4 KYA	0.991	8.036	109.4	0.3269	2.352	0.1928	0.1236
5 NEA	0.998	9.157	125.3	0.3449	3.530	0.1834	0.1100
6 BHE	0.996	10.98	145.1	0.4239	3.919	0.2387	0.1282
Mean		9.818	127.0	0.4070	3.675	0.2016	0.1150
SD		2.232	22.2	0.0901	1.125	0.0206	0.0103
CV		23	17	22	31	10	9

Table 5. Determined coefficients and R-square for the truncated model (bi-exponential function with a constant)

Subject	R^2	K_1	g_1	K_2	g_2	K_3
1 DGA	0.998	9.051	106.8	0.6328	5.260	0.2100
2 HSE	0.997	8.051	118.7	0.3732	4.823	0.1693
3 JCB	0.995	14.27	165.6	0.4190	4.086	0.2329
4 KYA	0.982	8.200	112.5	0.3461	3.147	0.1992
5 NEA	0.997	9.220	126.4	0.3627	3.747	0.1743
6 BHE	0.996	11.29	149.9	0.4533	5.119	0.2453
Mean		10.01	130.0	0.4312	4.364	0.2052
SD		2.385	23.0	0.1064	0.839	0.0306
CV		24	18	25	19	15

Table 6. Percent deviation of the coefficients of the truncated exponential function model from the tri-exponential function model

Subject	K_1	g_1	K_2	g_2	K_3
1 DGA	2.9	5.3	10.0	37.9	9.8
2 HSE	-0.8	-1.2	2.0	-14.1	-11.5
3 JCB	3.3	3.2	3.5	44.6	9.8
4 KYA	2.0	2.8	5.9	33.8	3.3
5 NEA	0.7	0.9	5.2	6.1	-5.0
6 BHE	2.8	3.3	6.9	30.6	2.8
Mean	1.8	2.4	5.6	23.2	1.5
SD	1.6	2.3	2.8	22.5	8.4

Except g_2, the coefficients estimated from the truncated exponential function model (1-day observation) agree well with the coefficients determined from the tri-exponential function model (9-day observation).

Table 7. Indicators describing quasi-equilibrium in the open mammillary system found in the subjects

Subject	Time when $IER_{1+2+3} = IER_1$	Time when IER_{1+2+3}/IER_1 takes maximum	Maximum of IER_{1+2+3}/IER_1	Time when IER_{1+2+3}/IER_1 takes 95 % maximum	IER_{1+2+3}/IER_1 at t = 1 day	Rem trace pool at 1
	day	day		day		
1 DGA	0.810	6.9	1.146	1.085	1.068	
2 HSE	0.593	5.8	1.070	0.644	1.062	
3 JCB	1.039	7.7	1.108	1.306	0.966	
4 KYA	1.128	7.8	1.129	1.533	0.964	
5 NEA	0.844	4.6	1.101	1.035	1.040	
6 BHE	0.757	6.9	1.097	1.091	1.056	
Mean	0.862	6.6	1.109	1.116	1.026	
SD	0.194	1.2	0.026	0.297	0.048	

Table 8. Comparison of rapidly exchanging Zn pool (EZP) by various methods (mg)

	Method 1	Method 2	Method 3	Method 4	Method 5	Meth
	Open three-pool*	Closed three-pool ($g_3 = 0$)	Constrained open three-pool ($g_3 = 0.115$)	Last term of tri-exponential	Simple extra-polation	One sp pla po
Interval	5 min - 9 d	5 min - 1 d	5 min - 1 d	5 min - 9 d	3 - 9 d	1
Subject 1 DGA	169	202	173	222	281	2(
Subject 2 HSE	192	251	174	222	-	2
Subject 3 JCB	162	182	153	200	218	1
Subject 4 KYA	171	213	179	220	228	2(
Subject 5 NEA	191	244	186	232	242	2
Subject 6 BHE	166	194	176	200	233	1
Mean	174	213	161	216	240	21
SD	14	27	19	11	22	2
CV	8	13	12	5	9	1

*Method 1 (open three-pool model) is considered as a norm.

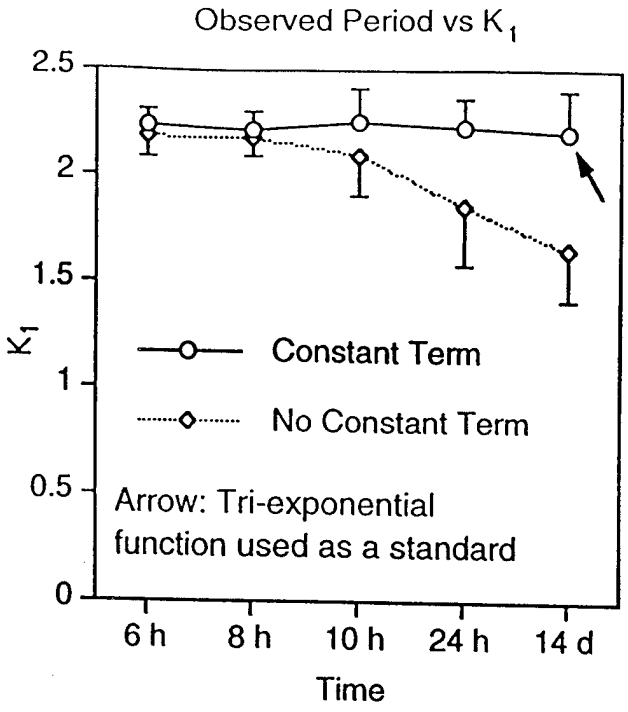
Table 9. Percent deviation of EZP estimates from the method 1 (open three-pool model)

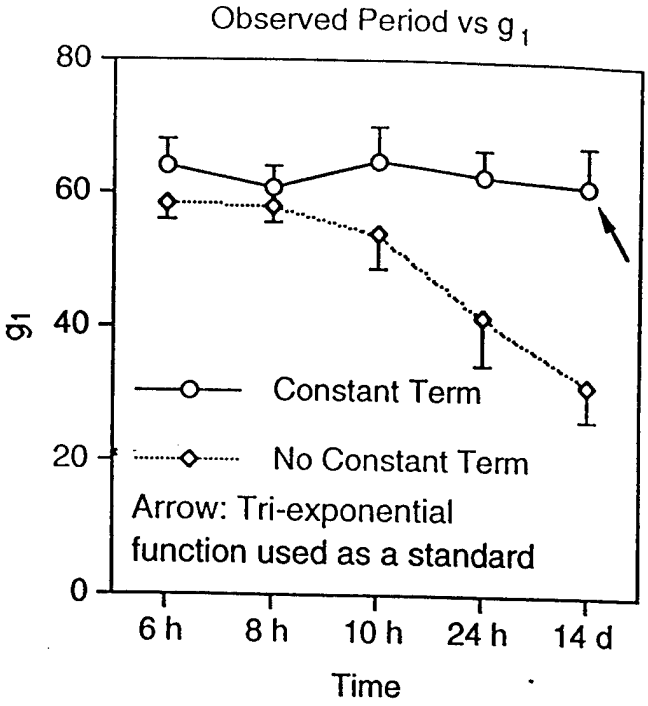
	Method 2	Method 3	Method 4	Method 5	Meth
Subject	Closed three-pool ($g_3 = 0$)	Constrained open three-pool ($g_3 = 0.115$)	Last term of tri-exponential	Simple extra-polation	One da plasm
Subject 1 DGA	19.5	2.4	31.4	66.3	21
Subject 2 HSE	30.7	-9.4	15.6	-	33
Subject 3 JCB	12.3	-5.6	23.5	34.6	12
Subject 4 KYA	24.6	4.7	28.7	33.3	20
Subject 5 NEA	27.7	-2.6	21.5	26.7	22
Subject 6 BHE	16.9	6.0	20.5	40.4	17
Mean	22.0	-0.7	23.5	40.2	21
SD	6.9	6.1	5.7	15.3	6.

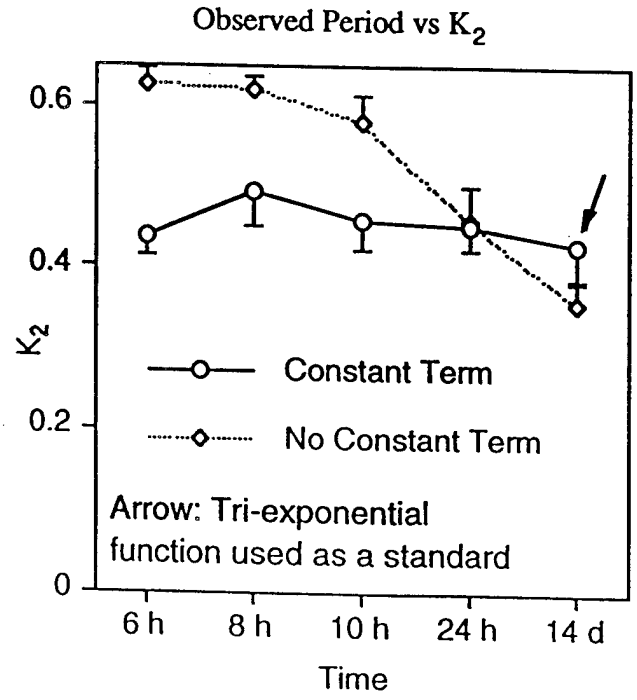
Table 10. Comparison of plasma Zn turnover rate (TR) determined from various models (mg/day)

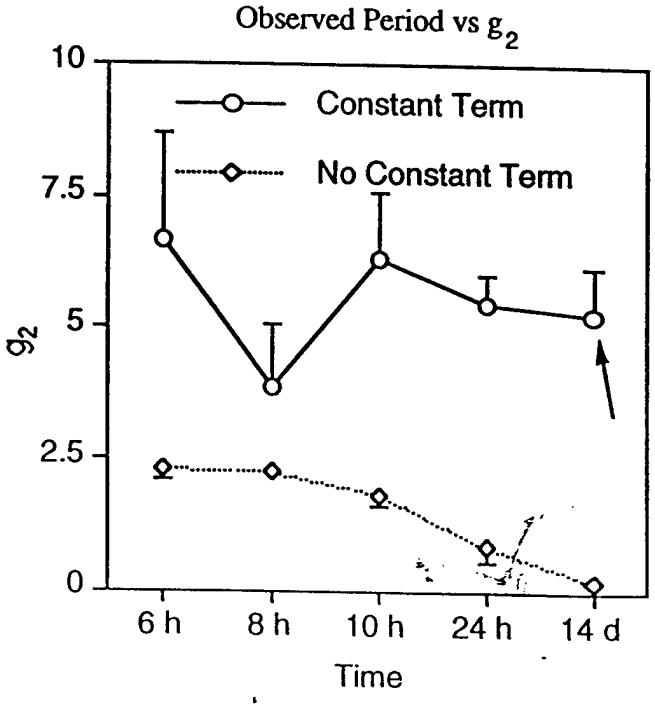
Models	Open three-pool	Closed three-pool ($g_3 = 0$)	Constrained open three-pool ($g_3 = 0.115$)	Initial two points (5 and 15 minutes)
Interval	5 min - 9 d	5 min - 1 d	5 min - 1 d	5 and 15 min
Subject 1 DGA	415	421	415	406
Subject 2 HSE	552	550	555	532
Subject 3 JCB	452	450	451	421
Subject 4 KYA	510	513	510	496
Subject 5 NEA	520	521	520	488
Subject 6 BHE	561	562	559	528
Mean	502	503	502	479
SD	57	56	58	53
CV	11	11	11	11

Analysis of Prasad's data









Circles in the figure are the estimated parameters. Bars are the asymptotic standard error.

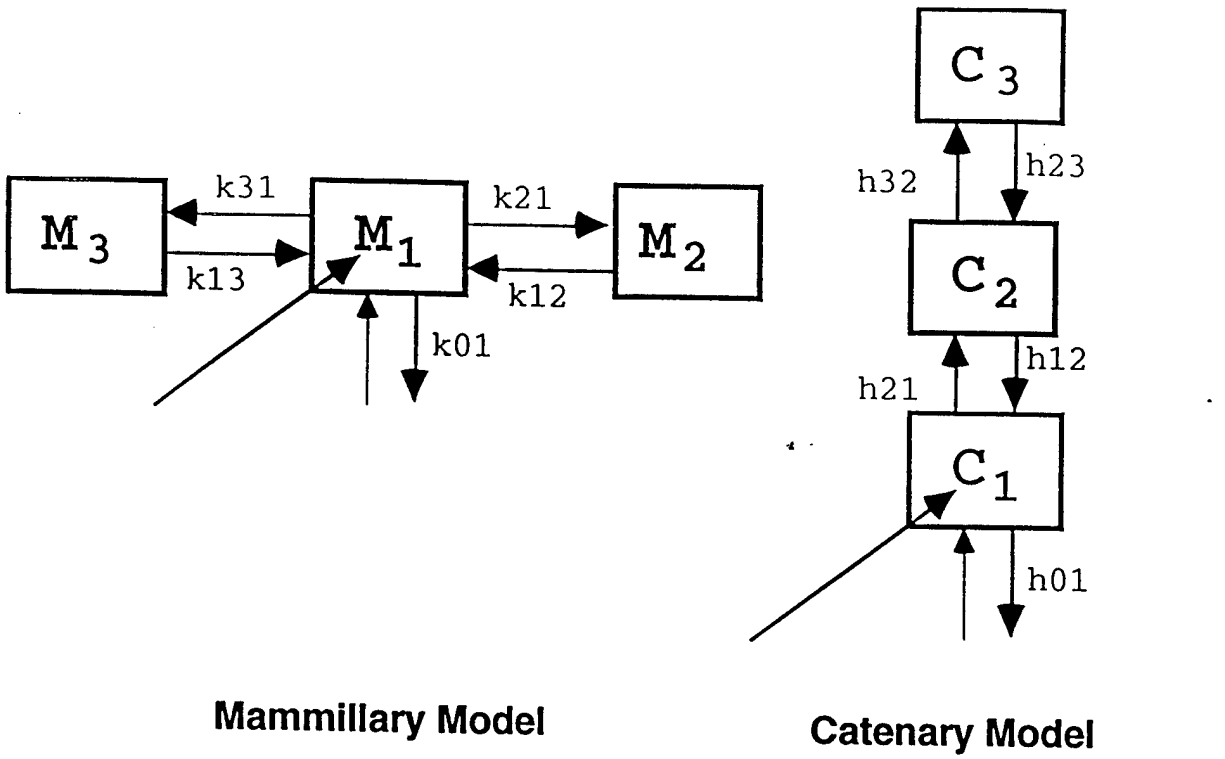


Figure 1. Mammillary and catenary models with three pools

M_1 corresponds to the central compartment in the mammillary model.
C_1 corresponds to the central compartment in the catenary model.
M_2 and M_3 are the peripheral pools.
C_2 and C_3 are the peripheral pools.

Mammillary model is a linear kinetic system which has noncentral or peripheral pools, each separately connected to a central pool without interconnection among peripheral pools.

Catenary model is a linear kinetic system which has several pools sequentially connected each other in the chain form.

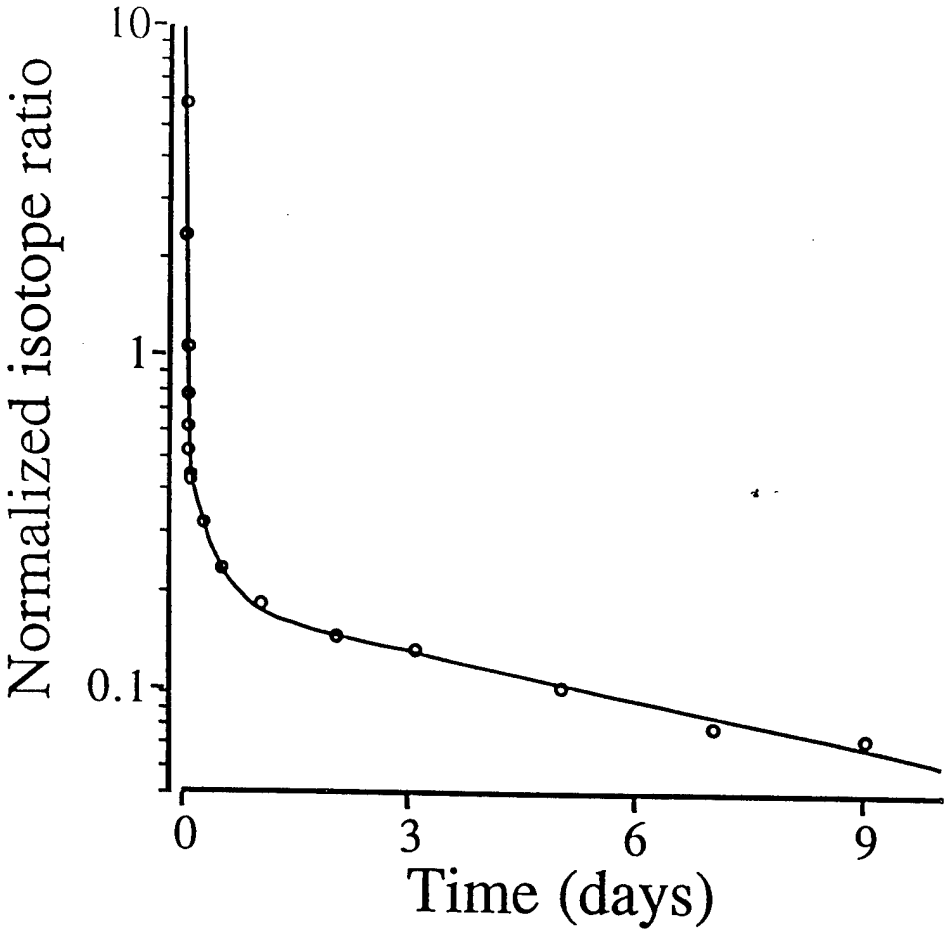


Figure 2. Illustration of the disappearance curve fitted to a triexponenti function (Data were obtained from Subject NE). Simplex minimization of residual square by nonlinear regression of SYSTAT Software using the following model equation:

Logarithm of normalized isotope ratio = $Log\left(K_1 e^{-g_1 t} + K_2 e^{-g_2 t} + K_3 e^{-g_3 t}\right)$

Acknowledgment

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References

Appendix 1.

Solving the mammillary model

Using Landaw et al's [Landaw, 1984 #808] algorithm

$$k_{11} = k_{01} + k_{21} + k_{31} = \frac{\sum_{i=1}^{3} K_i g_i}{\sum_{i=1}^{3} K_i}$$

Roots of the numerator of Laplace transformation of tri-exponential function give $k_{22} = k_{12} + k_{21}$ and $k_{33} = k_{13} + k_{31}$.

$$\gamma_j = k_{ij} k_{ji} = \frac{\sum_{i=1}^{3} K_i}{\sum_{i=1}^{3} \frac{K_i}{\left(k_{jj} - g_i\right)^2}}$$

For the single outlet or closed model, Q_2, Q_3, and 'k's are uniquely determined.

$$k_{22} = k_{12}$$

$$k_{33} = k_{13}$$

$$Q_1 = \text{Dose of tracer (mmol)} \Big/ \left(K_1 + K_2 + K_3\right)$$

$$Q_2 = \gamma_2 / k_{12}\, Q_1$$

$$Q_3 = \gamma_3 / k_{13}\, Q_1$$

$$\text{Rapidly Exchangeable Zn Pool (EZP)} = Q_1 + Q_2 + Q_3$$

where Q1 is the plasma Zn compartment (central compartment).

Appendix 2

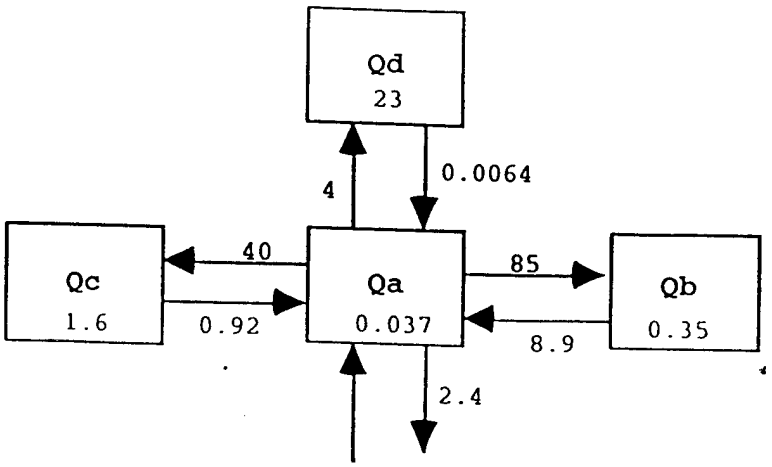


Figure 2-1. Miller's mammillary model

The unit for pool sizes are mmol. The unit for the rate constant is day^{-1}.

This section will be replaced by the following.

Numerical solution of Miller's Mammillary model

The following is the "Mathematica" statement that describes the numerical solution of Miller's mammillary model (open four-pool/single outlet).

```
NDSolve[{qa'[t] == 0.92 qc[t] + 0.0064 qd[t] + 8.9 qb[t]
- (2.4 + 40 + 4 + 85) qa[t],
qb'[t] == 85 qa[t] - 8.9 qb[t],
qc'[t] == 40 qa[t] - 0.92 qc[t],
qd'[t] == 4 qa[t] - 0.0064 qd[t],
qa[0] == 1, qb[0] == 0, qc[0] == 0, qd[0] == 0},
{qa, qb, qc, qd}, {t,0.001,10}]
```

where q_a [t], q_b [t], q_c [t] and q_d [t] correspond to the amount of tracer at time t in the respective pools (a, b, c and d) in the above figure.

'True' EZP = Qa + Qb + Qc = 0.037 + 0.35 + 1.6 = 1.987 mmol

EZP estimated from 3 - 9 day extrapolation to day 0 = 2.48 mmol (3 - 9 day Slope 0.1022 /day; true value 0.1106) (25 % higher)

1 day Plasma pool = 2.57 mmol (29 % higher)

Remaining tracer in the system 1 day later: 81.15 %

Quasi-Equilibrium Found in Miller's Illustration

Time (days)	IER_b/IER_a	IER_c/IER_a	IER_d/IER_a	IER_{abc}/IER_a
1	1.0614	1.0475	0.0131	1.0491
2	1.0230	1.1329	0.0223	1.1111
3	1.0217	1.1351	0.0314	1.1126
4	1.0216	1.1343	0.0414	1.1120
5	1.0215	1.1334	0.0523	1.1112
6	1.0214	1.1324	0.0644	1.1104
7	1.0213	1.1338	0.0776	1.1095
8	1.0212	1.1302	0.0920	1.1085
9	1.0210	1.1289	0.1078	1.1075

IER_{abc} is the isotopic enrichment in the sum of pools a, b and c.

Analytical solution of Miller's mammillary model

$$q_a(t) = 0.95449e^{-137.55t} + 0.030457e^{-3.56365t} + 0.0144254e^{-0.110596t} + 0.000628113e^{-0.00231993t}$$

$$q_b(t) = -0.630638e^{-137.55t} + 0.485133e^{-3.56365t} + 0.139504e^{-0.110596t} + 0.0060004e^{-0.00231993t}$$

$$q_c(t) = -0.279438e^{-137.55t} + 0.460832e^{-3.56365t} + 0.712891e^{-0.110596t} + 0.0273783e^{-0.00231993t}$$

$$q_d(t) = -0.0277582e^{-137.55t} + 0.0342478e^{-3.56365t} + 0.55378e^{-0.110596t} + 0.615786e^{-0.00231993t}$$

$$IER_a(t) = q_a(t)/Q_a = q_a(t)/0.037$$

$$IER_b(t) = q_b(t)/Q_b = q_b(t)/0.35$$

$$IER_c(t) = q_c(t)/Q_c = q_c(t)/1.6$$

$$IER_d(t) = q_d(t)/Q_d = q_d(t)/23$$

where q_a [t], q_b [t], q_c [t] and q_d [t] correspond to the amount of tracer at time t in the respective pools (a, b, c and d); IER_a, IER_b, IER_c and IER_d are the isotopic enrichment in pools a, b, c and d in the above figure.

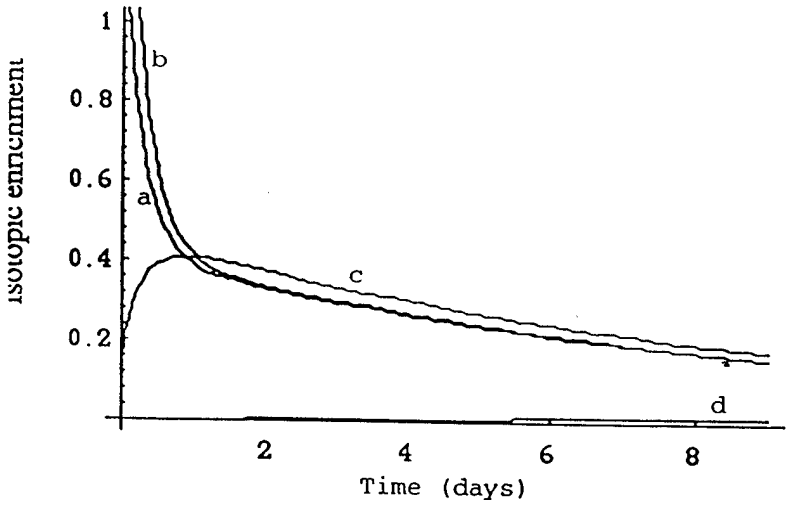


Figure 2-2. Isotopic enrichment inthe compartment of Miller's mammillar: model

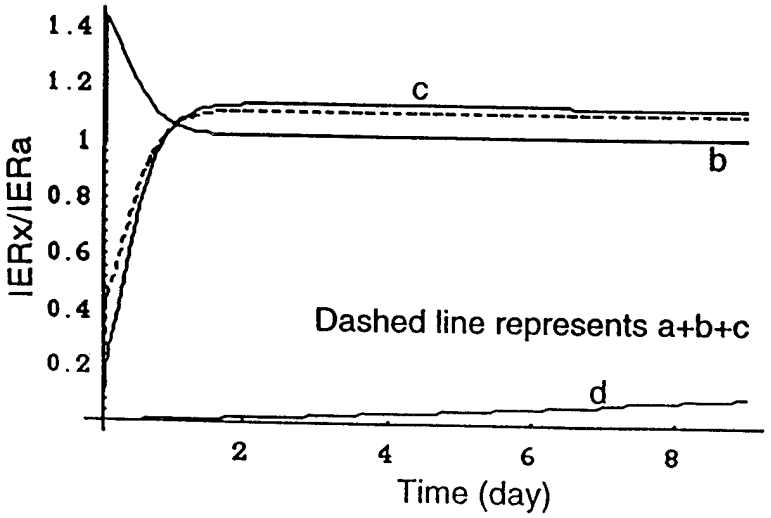


Figure 2-3. The ratio of isotopic enrichment in pool x to pool a (IER_x/IER_a x represents b, c and a+b+c. a+b+c represents the lump of pools a, b and c. About one day after the intravenous administration of tracer, pool b .and the lump of pools a, b and c reach 95 % maximum and are in the state of the quasi-equilibrium. Overshoot was observed in IER_b/IER_a.

Table 2-1. Indicators describing quasi-equilibrium in Miller's mammillar: model

Pool	Time when $IER_x = IER_a$	Time when IER_x / IER_a takes maximum	Maximum of IER_x / IER_a	Time when IER_x / IER_a takes 95 % maximum	IER_x / IER_a at t = 1 day

	day	day		day	
b	0.029	0.066	1.426	0.200	1.061
c	0.866	2.752	1.135	1.130	1.047
d	34.575	718.608	1.577	56.512	0.013
a+b+c	0.822	2.718	1.113	1.040	1.049

[1] a,b,c and d represent pools in Miller's mammillary model.

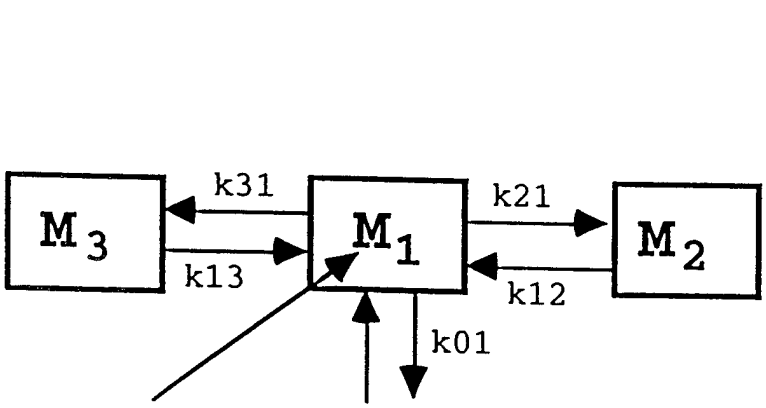
[2] a+b+c represents lump of pools a, b and c.

[3] x represents a, b, c or d.

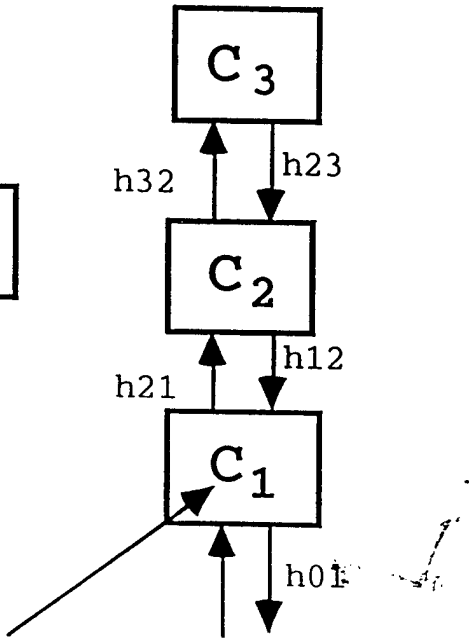
[4] IER represents isotopic enrichments.

Appendix 3.

Conserved parameters by matrix transformation of three pool systems of mammillary and catenary models



Mammillary Model



Catenary Model

When the disappearance curve is described by the tri-exponential function:

$$q_1(t) = K_1 e^{-g_1 t} + K_2 e^{-g_2 t} + K_3 e^{-g_3 t}$$

where $q_1(t)$ is enrichment in the central Zn compartment, 'K's are linear coefficients, 'g's are exponential coefficient and t is time in days. To give the order to the terms, $g_1 > g_2 > g_3$ is defined without loosing the generality.

Since the pool size of the central compartment is given by the iv dose (D, mmol) divided by the extrapolated enrichment.

$$q_1(0) = K_1 + K_2 + K_3 \quad : \textit{Extrapolated enrichment at } t = 0.$$

$$M_1 = \frac{D}{q_1(0)}$$

$$C_1 = \frac{D}{q_1(0)}$$

$$\therefore M_1 = C_1$$

where D is the intravenous dose of the tracer.

The sum of the rate constants from the central compartments is the initial slope of the disappearance curve divided by the $q_1(0)$.

$$k_{11} = k_{01} + k_{21} + k_{31}$$

$$= \frac{\sum_{i=1}^{3}(K_i g_i)}{\sum_{i=1}^{3}(K_i)}$$

$$h_{11} = h_{01} + h_{21}$$

$$= \frac{\sum_{i=1}^{3}(K_i g_i)}{\sum_{i=1}^{3}(K_i)}$$

$$\therefore k_{11} = h_{11}$$

$$F_{m11} = k_{11} M_1$$

$$F_{c11} = h_{11} C_1$$

$$\therefore F_{m11} = F_{c11}$$

where F_{m11} is the flux from the central compartment of the mammillary model and F_{c11} is that of the catenary model.

Ramakrishnan (1984) reported the indistinguishability between the mammillary and catenary models and reported the matrix transformation.

For the mammillary model,

$$A=\begin{bmatrix} -k_{11} & k_{21} & k_{31} \\ k_{12} & -k_{22} & 0 \\ k_{13} & 0 & -k_{33} \end{bmatrix}=\begin{bmatrix} -(k_{01}+k_{21}+k_{31}) & k_{21} & k_{31} \\ k_{12} & -k_{12} & 0 \\ k_{13} & 0 & -k_{13} \end{bmatrix}$$

$$q_m(0)=\begin{pmatrix} q_1(0) & 0 & 0 \end{pmatrix}$$

$$\frac{dq_m}{dt}=q_m\,A$$

At the steady state,

$$\frac{dM_1}{dt}=F_{m10}+k_{12}M_2+k_{13}M_3-k_{11}M_1=0$$

$$\frac{dM_2}{dt}=k_{21}M_1-k_{12}M_2=0$$

$$\frac{dM_3}{dt}=k_{31}M_1-k_{13}M_3=0$$

$$M_2=\frac{k_{21}M_1}{k_{12}}$$

$$M_3=\frac{k_{31}M_1}{k_{13}}$$

$$M_1+M_2+M_3=\left(1+\frac{k_{21}}{k_{12}}+\frac{k_{31}}{k_{13}}\right)M_1$$

For the catenary model transformed from the mammillary model,

$$B=\begin{bmatrix} -h_{11} & h_{21} & 0 \\ h_{12} & -h_{22} & h_{32} \\ 0 & h_{23} & -h_{33} \end{bmatrix}=\begin{bmatrix} -h_{11} & h_{21} & 0 \\ h_{12} & -(h_{12}+h_{32}) & h_{32} \\ 0 & h_{23} & -h_{23} \end{bmatrix}$$

$$q_c(0)=\begin{pmatrix} q_1(0) & 0 & 0 \end{pmatrix}$$

$$\frac{dq_c}{dt}=q_c\,B$$

$$=q_c\,T^{-1}AT$$

$$Select\ T^{-1}=P=\begin{bmatrix} 1 & 0 & 0 \\ 0 & \dfrac{k_{21}}{k_{31}+k_{21}} & \dfrac{k_{31}}{k_{31}+k_{21}} \\ 0 & \dfrac{k_{13}}{k_{13}-k_{12}} & \dfrac{k_{12}}{k_{12}-k_{13}} \end{bmatrix}$$

$$\frac{dq_c}{dt}=q_c\,PAP^{-1}$$

Matrix transformation from the mammillary model to the catenary model is as follows: (Remark. In his PAP^{-1} matrix (p. 382), "-" was deleted (typographically?) from the 3, 3rd entry.)

$$B=PAP^{-1}$$

$$=\begin{bmatrix} -k_{11} & k_{31}+k_{21} & 0 \\ \dfrac{k_{31}k_{13}+k_{21}k_{12}}{k_{31}+k_{21}} & -\dfrac{\left(k_{31}k_{13}^2+k_{21}k_{12}^2\right)}{k_{31}k_{13}+k_{21}k_{12}} & \dfrac{k_{21}k_{31}\left(k_{12}-k_{13}\right)^2}{\left(k_{21}+k_{31}\right)\left(k_{31}k_{13}+k_{21}k_{12}\right)} \\ 0 & \dfrac{k_{12}k_{13}\left(k_{31}+k_{21}\right)}{k_{31}k_{13}+k_{21}k_{12}} & -\dfrac{k_{12}k_{13}\left(k_{31}+k_{21}\right)}{k_{31}k_{13}+k_{21}k_{12}} \end{bmatrix}$$

At the steady state,

$$\frac{dC_1}{dt}={}_cF_{10}+h_{12}C_2-h_{11}C_1=0$$

$$\frac{dC_2}{dt}=h_{21}C_1+h_{23}C_3-(h_{12}+h_{32})C_2=0$$

$$\frac{dC_3}{dt} = k_{32}C_2 - k_{23}C_3 = 0$$

$$C_2 = \frac{h_{21}C_1}{h_{12}}$$

$$= \frac{(k_{21}+k_{31})^2}{k_{12}k_{21}+k_{13}k_{31}} M_1$$

$$C_3 = \frac{h_{32}C_2}{h_{23}}$$

$$= \frac{(k_{12}-k_{13})^2 k_{21}k_{31}}{k_{12}k_{13}(k_{12}k_{21}+k_{13}k_{31})} M_1$$

$$\therefore C_1 + C_2 + C_3 = \left(1 + \frac{k_{21}}{k_{12}} + \frac{k_{31}}{k_{13}}\right) M_1$$

$$= M_1 + M_2 + M_3$$

Summary of the conserved parameters

1. Pool size of the central compartment (plasma Zn pool)
2. Sum of the rate constant from the central compartment
3. Flux from the central compartment (plasma Zn turnover rate)
4. Sum of the pool sizes (so-called rapidly exchangeable Zn pool, EZP)

March 26, 1997

Zinc Kinetics: (Progress during April through June, 1997)

Isotope ratio analysis by ICP-MS for the determination of Zn disappearance rates and pool sizes in plasma and urine samples were completed for the following subject numbers:

194	**221**	**233**	**167**	**225**
203	**228**	**226**	**189**	**258**

Two manuscripts (preliminary drafts) on Zn kinetics were also prepared for publications. Thus far Zn kinetics have been performed on 42 study subjects plus 6 male participants in the initial pilot study.

Army Grant Report - July 1, 1997

Since the previous report, the white cell isolation procedure has been performed on 15 subjects. Although performed by a different technical assistant, there was no difficulty with the procedure. In 4 individuals, the granulocyte count has been very low, perhaps due to some clumping of the cells, and therefore an incomplete sample obtained for counting and zinc determination. Discussion with Dr. Beck whose published procedure is used, indicated that this is a sporadic finding, the reason for which has not been identified. A critical examination of the various steps for loss of white cells is being pursued. In other subjects the granulocyte count was similar to previously reported, and the purity of the cell fractions comparable.
The zinc analysis on the various cell fractions is pending, as this is performed in batches, in order to contain the ever present potential for environmental contamination. The use of an electrodeless discharge zinc lamp, has improved baseline drift associated with the hollow cathode lamp in the past.
It is planned to measure magnesium in the cell fractions, to check on the constancy of this, and the possibility of using this as a reference value.

Difficulty with serum beta-hydroxy-butyrate kits from Sigma Chemical Co. has been resolved, and none of the 24 specimens stored at -70° C had an elevated level.

Appendix V

	This Quarter 3/23/97 to 6/22/97	Entire Study	Scheduled	Need to schedule
Completed Telephone Questionaire	75	322		24
Completed Phase 1 Screening	37	118	1	
Completed Phase 2 Isotope assessmen	16	42	9	0
Completed Phase 3B	12	26	2	4
Completed Phase 3C	4	13	1	10
Completed Study	4	7	1	N/A
Subjects not eligible	11	29	N/A	N/A
Subjects dropped	2	38	N/A	N/A